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OPTIMIZATION OF DOUBLED HAPLOID PRODUCTION IN BURLEY TOBACCO (*Nicotiana tabacum* L.)

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OPTIMIZATION OF DOUBLED HAPLOID PRODUCTION IN BURLEY TOBACCO (*Nicotiana tabacum* L.)

DISSERTATION

A dissertation submitted in partial fulfillment of the
requirements for the degree of Doctor of Philosophy
in the College of Agriculture, Food and Environment
at the University of Kentucky

By

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Director: Dr. Robert D. Miller, Professor of Plant and Soil Sciences

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ABSTRACT OF A DISSERTATION

OPTIMIZATION OF DOUBLED HAPLOID PRODUCTION IN BURLEY TOBACCO (*Nicotiana tabacum* L.)

Doubled haploidy (DH) is a plant breeding technique that is often utilized by plant breeders to minimize the time required to reach homozygosity in breeding lines. The first objective of this study was to compare two methods of generating DH lines in tobacco (*Nicotiana tabacum* L.). Inbred burley tobacco varieties TN 90LC and GR 149LC were used to produce both androgenic derived doubled haploids (ADDH) and maternally derived doubled haploids (MDDH). The relative agronomic performance of TN 90LC and GR 149 LC ADDH and MDDH lines was compared when used either as pure-line cultivars or when used for the production of the KT 204LC and TN 97LC hybrid cultivars, respectively. The ADDH method was more efficient than the MDDH method in generating large numbers of haploid plants. On average the ADDH TN 90LC population was statistically inferior to the inbred TN 90LC for several agronomic traits; this inferiority of the ADDH method was not observed in the GR 149LC populations. For both genotypes, the MDDH populations were comparable to the inbred parental genotypes. The ADDH method was inferior for TN 90LC, but several individual TN 90LC ADDH lines were equal or superior to the inbred source. The agronomic variability observed in both ADDH and MDDH lines was decreased when they were used to produce hybrid cultivars. Less variation was observed in the DH-derived hybrids KT 204LC and TN 97LC compared to the ADDH and MDDH TN90LC and GR149LC parental lines, respectively. The significant inferiority of ADDH TN 90 lines in comparison to inbred TN 90LC was not observed in the ADDH derived KT 204 population compared to KT 204LC. The second objective of this study was to compare DH Lines derived from an F_1 breeding population versus DH lines derived from a segregating F_2 population where plants used for DH were pre-screened for quantitatively inherited resistance to soil-borne diseases black shank (*Phytophthora nicotianae*) and/or *Fusarium* wilt (*Fusarium oxysporum* f. *nicotianae*). There was a clear difference in susceptibility to black shank between the F_1 and F_2 derived DH populations, both in terms of average disease incidence, and more importantly, in the percentage of individual lines displaying high disease resistance. For two different burley crosses, DH lines derived from the F_1 generation were considerably more susceptible to black shank than DH lines derived from the F_2 generation. No differences in the incidence of *Fusarium* wilt were observed between DH lines of F_1 and F_2 generations; this was likely due to low overall disease incidence. Although delaying the DH process in tobacco from the F_1 to the F_2 generation could add time to the development of homozygous breeding lines, the delay may be offset by having to screen fewer finished DH lines to identify superior lines.

KEYWORDS: Tobacco breeding, doubled haploidy, black shank, *Fusarium* wilt

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CHAPTER 1: Introduction

Tobacco (*Nicotiana tabacum* L.) is cultivated worldwide as one of the most relevant annual non-food crops. World tobacco production in 2011 was around 7.38 million metric tons with more than 80% produced by China, Brazil, India, United States of America (US) and Malawi (FAO, 2013). As with any other agricultural crop, tobacco faces local and widespread challenges every year with regard to the field growing cycle. Farmers, industry and consumers have specific demands from the product that they are interested in. However, these demands are usually unique within these differing sectors.

Plant breeders use a variety of tools and methods to hasten the process of generating reliable solutions to attend to industry demand. Doubled haploid (DH) strategy is one of the most promising techniques to efficaciously produce highly homozygous diploid plants from heterozygous sources in a short period of time (Belogradova et al., 2009). The first report of haploids in a plant species dates back to the 1920s, and the initial successful DH production protocol is from half a century ago. To date, haploids have been used in over 200 plant species for breeding purposes and genetics studies (Touraev et al., 2009).

Tobacco production is affected by many factors, including soil-borne diseases. Due to the importance of resistant varieties in minimizing losses in tobacco resulting from the incidence of diseases, and the frequent use of DH in developing resistant cultivars, the objective of this research project was to determine the optimal integration of DH breeding methods into the development of breeding lines with targeted disease resistance traits. The research project consisted of two studies, with the combined results anticipated to maximize the overall efficiency of using DH in the development of new tobacco cultivars.

The objective of the first study was to compare two methods of generating DH materials in a tobacco breeding program, either for use as self-pollinated pure line varieties or for the development of homozygous parental lines for use in the development of hybrid tobacco cultivars. Androgenic derived haploid (ADH) lines, obtained via anther culture, and maternally derived haploid (MDH) lines, obtained via inter-specific hybridization with

Nicotiana africana Merxm. (2n = 46), were the methods compared for this study. The comparison of these methods included: a) the relative efficiency of generating ADH versus MDH lines; and b) the relative agronomic performance of androgenic derived double haploids (ADDH) versus maternally derived doubled haploids (MDDH) lines, either as pure-line varieties or when used for the production of hybrid cultivars.

The objective of the second study was to determine the most effective breeding generation, F_1 versus F_2, in which to develop haploid lines in cases where quantitatively inherited resistance to soil-borne diseases is the primary objective. Because one of the objectives of using DH procedures is to shorten the period of time required to achieve homozygosity, the F_1 generation is typically utilized to produce haploid plants. The resultant DH lines are then evaluated for disease resistance. Because the level of disease resistance is genetically fixed, selection for resistance is restricted to differences between DH lines rather than individual plants. As a result, a large number of haploid lines usually need to be evaluated in order to identify lines that have superior disease resistance. In cases where the primary objective is to maximize the level of quantitatively inherited disease resistance, it may be more effective to delay the development of haploid plants until the F_2 generation. Although the attainment of homozygosity would be delayed by one generation, by growing the F_2 population in the presence of the disease pathogen, plants displaying superior levels of disease resistance could be selected for the generation of haploid lines. By pre-screening for disease resistance, it may be possible to more efficiently identify haploid lines having a high level of resistance to the targeted disease.

CHAPTER 2: Literature Review

Tobacco

The genus *Nicotiana* is comprised of 76 naturally occurring species, most of which are native to the Americas (Knapp et al., 2004). Exceptions are *N. africana* (autochthonous to Namibia) and the *Suaveolentes* section, indigenous to Australia and surrounding islands (Kelly et al., 2013). *N. tabacum* is native to South America and is thought to have originated over 200 million years ago in the eastern slopes of the Andes ridge. The species is a classic amphidiploid (2n = 4x = 48) and apparently arose by the union of unreduced gametes of *N. sylvestris* Speg. & Comes (2n = 24), the maternal genome donor, and *N. tomentosiformis* Goodsp (2n = 24) (Leitch et al., 2008).

The most feasible hypothesis about the dispersion of tobacco from its center of origin is linked to Christopher Columbus' voyages, when Europeans became aware of the existence of the American continent, in 1492. At that time, natives of the newly discovered continent used tobacco to inhale the smoke from burning leaves, or to chew its leaves. Before long, tobacco became a companion and distraction for mariners onboard the European fleets during navigation, who spread it along the ports where they anchored (Tso, 1990). Currently, tobacco is a very cosmopolitan crop, with major producers and consumers present in Asia, South and North America, Europe and Africa.

The primary use of tobacco leaves are for production of cigarettes, cigars and smokeless products. Differentiation of commercial tobacco types are mainly based on curing characteristics. The two major components in blended cigarettes are the major tobacco types grown in the US, which are the flue-cured or "Virginia" type, and burley and Maryland which are included in the light air-cured type. Additionally, the US grows dark air-cured, cigar, fire-cured and sun-cured types, but does not produce a few other classes including oriental tobacco, which are important worldwide (Sullivan, 2004).

Tobacco was the most important commodity in the colonial period of the US. Commercial production started in Virginia (VA) in the early 1600's, comprising the most prominent exported product at the time. It rapidly

expanded to Maryland (MD) and, in the late seventeenth century, into North Carolina (NC), Kentucky (KY), Tennessee (TN), Ohio (OH) and to others states (Tso, 1990). There is a clear regional division of production areas with regard to tobacco types in the US, especially across the Appalachians. On the eastern side of Appalachian Mountains, flue-cured tobacco is the prevalent type. In the western region of the mountains, which includes the states of TN and KY, air-cured and fire-cured tobacco are predominant.

Modern burley tobacco originated in 1864 when a field of “red burley” was set out in Higginsport, OH using seeds from Bracken County, KY. The entire crop appeared pale and sick, but once it was air-cured a white to yellow shade was noted in cured leaves, originating the name “white burley” or simply “burley”. The special strain quickly became popular, replacing older varieties like Red Burley, Standup and Twist Bud (Jahn, 1954). Burley production was very dynamic over the centuries with regard to regions in which it is produced. Since its origin, KY traditionally has led in the production of burley, followed by eastern TN and southern OH. The worldwide production of burley in 2012 was greater than 800,000 metric tons, representing 12.3% of all types of tobacco produced. Today’s largest burley producer is Brazil, followed by Malawi, US, China, Argentina and India (AFUBRA, 2014).

Doubled Haploids

For hybrid seed production in a large number of plant species, a fast and inexpensive method to obtain homozygous lines is a priority. The use of classical inbreeding and selection techniques to obtain pure lines is relatively expensive and time consuming, requiring several years to achieve homozygosity in plants (Seguí-Simarro, 2010). Doubled haploidy as a plant breeding method was born in 1964 following the establishment of an efficient protocol for androgenic derived doubled haploid (ADDH) production in the weed species *Datura innoxia* Mill. The technique was soon implemented to others crops species, including *N. tabacum* (Bourgin and Nitsch, 1967).

Production of DH parental lines is often used to significantly reduce the amount of time required to achieve homozygosity in tobacco breeding

populations. Schnell (1980) pointed out that the development and release of flue-cured tobacco cultivars from heterozygous germplasm requires ten or more years using common inbreeding methods; DH could save up to four years in the inbreeding process. Additionally, the absence of intra-allelic interactions due to complete homozygosity favors the identification of advantageous genotypes. The number of individuals to be screened in an attempt to find specific combinations of desirable genes is reduced drastically with the use of DH, when compared to screening of heterozygous populations (Chalyk, 2012). While time efficiency is the primary objective of DH strategy, Li et al. (2013) reported that for wheat the genetic gain per year for yield was lower for lines developed via conventional breeding methods compared to lines developed using DH breeding techniques. The advantages of DH techniques are particularly pronounced in crops where DH lines are efficiently produced, and where just one generation can be grown every year, limiting the effectiveness of conventional methods.

Maize is the most significant commercial crop in which DH techniques have been employed, but the method is successfully used in several other important crops. In some species like tobacco, maize, triticale and wheat, both ADH and MDH methods are suitable for developing DH lines, even though one of the methods may be more responsive in comparison to the other. In other plants (potato, onion, sugar beet) just one method is appropriate for induction of haploids. Other than the use as a plant breeding strategy, DH are also used for mutation studies, genetic mapping and plant transformation (Touraev et al., 2009).

Tobacco breeders and geneticists began to apply the techniques for ADDH production in burley and flue-cured tobaccos as soon they overcame earlier difficulties in adapting protocols from others species. By the time the use of ADDH method was well established, a maternally derived doubled haploid (MDDH) technique emerged as an alternative for haploid production in tobacco. The MDDH technique involves the interspecific hybridization between *N. tabacum* and *N. africana* (Burk et al., 1979). ADDH and MDDH are currently the common methods used to originate DH in tobacco, both having advantages and disadvantages with regard to plant vigor and yield, techniques needed, equipment, and time required for production of DH lines.

The most prominent reported disadvantage with regard to using DH techniques in tobacco is the unsatisfactory agronomic performance. ADDH lines have been shown to be inferior to the cultivars from which they were derived when compared for overall agronomic performance (Arcia et al., 1978; Deaton et al., 1982; Deaton et al., 1986a). Although MDDH performed better than ADDH lines, neither displayed equal yielding ability compared to the selfed progenies of the parental line (Wernsman et al., 1989; Nielsen and Collins, 1989). The unsatisfactory agronomic performance of ADDH lines could be due to high levels of homozygosity in DH plants (Niemirowicz-szczytt, 1997), genetic modifications as a result of anthers culture (Wernsman et al., 1989) or more specifically, DNA amplification as consequence of the haploid regeneration process (Reed et al., 1994).

Poor performance is not an exclusive problem in tobacco. In a comparison between modified single seed descent, conventional mass selection, and DH breeding methods for maize inbred line development, the DH had the lowest proportion out of ten best lines for grain yield and yield:moisture ratio (Jumbo et al., 2011). Successful improvements for the production of tobacco DH have been made over the past decades, increasing the yield of ADDH lines. Even though on average ADDH lines did not perform as well as conventional inbred lines, some individual lines within the DH population would equate and even surpass the yield of conventional inbred populations (Nielsen and Collins, 1989).

Although several studies report the inferiority of ADDH versus MDDH techniques for the production of homozygous inbred lines or varieties, studies have not been conducted to clearly determine whether the differences are detectable when ADDH or MDDH lines are used only for the development of hybrid cultivars, which are typically now used for commercial tobacco production. Included in the objectives of this research is the evaluation of hybrid cultivars originated as result of a cross between diploid and DH lines, to estimate whether the DH characteristics of parental lines are transmitted to the F_1 hybrid lines.

Androgenic Derived Haploids

Soon after protocols for the production of haploids derived from anthers were established in *D. innoxia*, studies using the technique in tobacco plants were performed initially in Europe (Bourgin and Nitsch, 1967) and Japan (Nakata and Tanaka, 1968). In the US, initial studies of ADH were reported in the early 1970's in flue-cured (Burk, 1970) and burley tobacco (Kasperbauer and Collins, 1972). ADH techniques proved to be very suitable for the production of haploids and quickly became the primary method for DH production in tobacco.

Anther derived haploid plantlets are the result of a redirection of microspores from the normal gametophytic pathway to sporophytic development through gametic embryogenesis (Sood et al., 2013). Haploid formation is an ecological mechanism of adaptation and is a consequence of environmental stresses. In the process of inducing haploids *in vitro*, the culture conditions to which anthers are subjected triggers physiological stress responses and changes in gene expression in the microspores, resulting in nuclear and cytoplasmic differentiation, leading to totipotency and the ability to form embryos. The redirection of microspores into embryonic response is influenced to a great extent by growth conditions of donor plants, pretreatment of flower buds, genotype, developmental stage of pollen, and the composition and conditions of culture medium (Germanà, 2010).

The interaction of endogenous and exogenous factors will determine the cytological and genetic responses of microspores. Accumulation of heat shock proteins (HSP) in response to heat stress in *Brassica napus* preceded dedifferentiation of microspores towards a sporophytic pathway (Seguí-Simarro et al., 2003). In a study utilizing tobacco, pepper (*Capsicum annuum* L.) and *B. napus*, Testillano et al. (2000) demonstrated that heat stress triggers signal transduction of mitogen-activated protein kinase (MAPK) additionally to enrichment of HSP70. Distinct and complementary mechanisms are differentially activated in the microspore, depending on the species and the nature of the environmental stress.

Significant reductions in yield of ADDH plants, when compared to inbred lines, have been extensively reported in burley and flue-cured tobacco

(Arcia et al., 1978; Deaton et al., 1986; Nielsen and Collins, 1989). This is the most prominent agronomic disadvantage of the ADDH method compared to MDDH and inbred lines. Several studies in tobacco suggested that the yield reductions could be associated with the negative effect of somaclonal variation during the haploid induction process and tissue culture procedures (Schnell and Wernsman, 1986; Wernsman et al., 1989). Somaclonal and gametoclonal variation are changes in the genetic make-up of an organism, which could be deleterious or advantageous. Considering a normal distribution, if many DH plants are produced and evaluated, the best performing lines could generate acceptable inbred lines in terms of agronomic traits.

Maternally Derived Haploids

There are three pathways for producing the so called maternally derived haploids: 1) the development of embryos, derived from the embryo sac, from cultured unfertilized isolated ovules; 2) interspecific hybridization followed by chromosome elimination, generally of the paternal parent; and 3) the process of parthenogenesis, in which there is growth of an embryo by apogamy, semigamy or pseudogamy. In tobacco, the most common method used is the interspecific hybridization, sometimes referred to as wide hybridization crosses or *bulbosum* method, since it is broadly used for MDH production in barley (*Hordeum vulgare* L.) (Palmer et al., 2005). Even though the use of the term gynogenesis is controversial among plant biologists, since it is specifically applied for haploid regeneration from unpollinated gametophytes, it is broadly used for any method involving female gametophytes (Bohanec, 2009).

The first female gametophyte haploid plant was identified in 1922, derived from *Datura stramonium* (Blakeslee et al., 1922); the initial *N. tabacum* MDH plant was reported a couple of years later (Clausen and Mann, 1924). A half century elapsed before the initial successful *in vitro* protocol for MDDH production was accomplished on barley (Kasha and Kao, 1970; San Noeum, 1976). This was the beginning of MDDH as a plant breeding strategy, because previous difficulties in inducing haploidy and identification of haploids

were an impediment for convenient use of this method. MDH techniques are primarily used in crops in which ADH is not a suitable method, like barley, wheat and potato.

A protocol for the efficient production of MDH in tobacco was developed in 1979, as an alternative for the ADH method. The use of *N. africana* pollen to pollinate *N. tabacum* allowed a straight forward morphological identification of *N. tabacum* haploids. Even though the majority of the seedlings from the interspecific cross senesce before formation of true leaves, 0.25 to 1.42% of the seeds germinate; these seedlings are comprised of compatible interspecific F_1 hybrids or haploids plants of *N. tabacum*. This is a suitable haploid production methodology, especially when there are restrictions in the use of ADH techniques (Burk et al., 1979).

Even though haploids can be obtained from both MDH and ADH methods in tobacco, the latter constitute a more efficient procedure for DH production. A very relevant factor in the induction of haploids *via* gynogenesis is the specificity of genotypes. Differential response in yield of haploids varying from 0 to almost 200% was observed in studies with several genotypes of onion (Bohanec, 2009). Nunes (2009) showed that burley generated, on average, 1 haploid plant per 2,100 seeds while in flue-cured tobacco this ratio was 1 haploid per 8,300 seeds. Burk et al. (1979) reported that the percentage of haploids among all surviving seedlings of three different flue-cured varieties crossed with *N. africana* varied from 4.23 to 15.94%. Our own experience producing MDH from two different burley tobacco lines showed a differential cultivar response in terms of number of haploids produced.

The number of MDH plantlets produced is dependent primarily on the genotype and environmental conditions to which the cross with *N. africana* and seed capsule development is exposed, but data comparing response of tobacco genotypes are very scarce. Another obstacle is the undesirable influence of the paternal genome in the phenotype of haploid plants, resulting from incomplete chromosome elimination during embryogenesis. Even though the expected outcome is a haploid plant containing only the female genome, retention of whole chromosomes or part of chromosomes from the male parent is frequently reported in several plant species (Kynast et al., 2001).

Optimal Generation to Induce Haploids

Choosing the best generation in which DH should be induced is a paradigm when considering quantitatively inherited traits in plants. The most efficient pathway is to produce haploids as early as possible, i.e. derived from the F_1 generation. On the other hand, the most efficacious way is to delay the induction process and advance as many generations as possible, allowing more recombination events and permitting phenotypic selection. Information about the best generation in which tobacco haploids should be induced is limited; it has been most widely studied in maize and small grains.

A simulation study in maize by Bernardo (2009) comparing recombinant inbred lines (RIL), F_1 and F_2-derived DH lines showed that F_1-derived plants sustain the lower long-term selection response, with F_2-derived plants intermediate between RIL and F_1-derived DH. The F_1-derived DH lines are expected to show greater response per unit of time (efficiency), while RIL have greater long-term response per cycle. Li et al. (2013) evaluated genetic gains for yield and adaptation traits in wheat comparing a selected bulk method (SELBLK) with F_1 and F_3-derived DH lines. It was reported that the F_1-derived DH lines showed higher genetic gains for yield but lower gains for the adaptation trait, which could be explained by the lower selection intensity applied to yield and the time efficiency of the DH strategy. The performance of DH lines in the study were different from Bernardo (2009), because the former study considered the possibility of advancing two generations per year, making the DH lines economically and genetically less efficient than SELBLK.

Charmet and Branlard (1985) reported no differences in most of the yield components in triticale (*X triticosecale*) between DH lines derived from F_1 plants and selfed lines inbred through the single seed descent method. The findings indicate that similar ranges of recombination of several generations of selfing can be achieved in F_1-derived DH, with the results suggesting that there is no need to delay the induction of haploids until the F_2 generation. Jannink and Abadie (1999) showed that DH provided the biggest short-term genetic gain, but it was inferior to the single seed descent method in the long-term. The complexity in determining the genetic and economic efficiency of most breeding methods are based on the interaction of a considerable

number of factors, such as plant species, number of generations per year, number of alleles controlling the trait, linkage, selection intensity and the frequency of alleles.

Quantitatively Inherited Soil-borne Diseases in Tobacco

Soil-borne diseases are associated with major losses in all types of tobacco grown in the US and worldwide. Oomycetes, fungi and bacteria are the common microorganisms responsible for damaging tobacco crops. Breeding resistant tobacco varieties is one of the most effective control measures to minimize risks associated with diseases. Frequently, the mechanisms of resistance to certain diseases and pathogen races are quantitatively inherited; i.e. controlled by several genes. The interaction of multiple genes controlling one trait may increase the difficulty of the breeding process by increasing the complexity to achieve highly resistant varieties, compared to traits controlled by a single gene.

One of the objectives of this Ph.D. study was to assess the level of quantitatively inherited resistance to two diverse diseases, black shank and *Fusarium* wilt, in DH lines originating from F_1 versus F_2 generations. Black shank is a disease caused by the oomycete *Phytophthora nicotianae* (Van Breda de Hann), which primarily infects roots, but also affects stalks and leaves of all types of tobacco, causing stunting and plant death at any stage of development (Shew et al., 1991; Gallup et al., 2006). In the US, black shank is the most destructive disease in burley and dark tobacco (Pearce et al., 2013) and it causes considerable losses in flue-cured and cigar tobaccos. Tobacco-producing regions in warmer climates worldwide tend to have the most severe problems with the disease, but black shank is virtually ubiquitous in all tobacco areas (Lucas, 1975; Shew et al., 1991).

Three races of *P. nicotianae* have been identified in the US (Apple, 1962) but the most important are race 0 and race 1. Race 0 is considered the wild type and occurs in all tobacco-growing areas, being the most virulent race for burley and predominant in flue-cured tobacco in NC since 1931(Shew et al., 1991). In 1954, race 1 was reported in the US in breeding lines of burley tobacco in Kentucky (Apple, 1962). The increase in predominance of

this race has resulted from the use of cultivars containing *Php* and *Phl* genes, which are single gene mechanisms conferring complete resistance to race 0 (Sullivan et al., 2005). *P. nicotianae* race 3 was first reported in Connecticut from a cigar-wrapper tobacco (McIntyre and Taylor, 1978). In 2010 this race was reported in NC, based on field samples and root inoculations using varieties carrying the *php* gene (Gallup and Shew, 2010). Race 2 was described in South Africa, but is not considered epidemic for tobacco (Prinsloo and Pauer, 1974).

Black shank can also affect tobacco plants still in the seedbeds causing damping-off of seedlings. In the field, the first visual symptom is wilting of the plant during the warmest part of the day, evolving to drooping and yellowing of the leaves. Typical symptoms of the infection are necrosis of the stem pith, which appears dry, brown or black in color developing from the basal to apical region. High soil moisture and warm temperatures, sometimes favored by dry periods, increase infection rates and evolution of symptoms (Shoemaker and Shew, 1999).

The most effective control measure for black shank is the use of resistant cultivars, because of the potential for cost-effective protection from genetic resistance. Even though there are no varieties having complete resistance to both race 0 and race 1, satisfactory results are obtained through a combination of different cultivars with single-gene resistance and cultivars having high quantitatively inherited resistance (Sullivan et al., 2005).

Another soil-borne disease for which quantitatively inherited resistance is utilized is *Fusarium* wilt, which affects a broad range of plant species worldwide. It usually infects scattered tobacco plants in the field in all types of soil, but the incidence is often associated with wet areas and sandy soils, such as river bottoms. In the US, *Fusarium* wilt was first reported in the state of Maryland in 1921 and it is widely dispersed in all tobacco areas (Shoemaker and Shew, 1999). The disease is caused by the ascomycete *Fusarium oxysporum* f. *nicotianae* (J. Johnson) W.C. Snyder & H.N. Hansen 1971(anamorphic Gibberella).

The *formae speciales* (ff.) and races of *F. oxysporum* infecting tobacco have not been defined in detail to date. Lucas (1975) mentioned studies in which the species was considered as different races of *F. oxysporum* f.

batatas and f. *vasinfectum*, because researchers were not able to find isolates specific to *N. tabacum*. Clark et al. (1998) defended the designation of *F. oxysporum* f. *nicotianae* because part of the confusion is due to differential virulence of distinct isolates of *F. oxysporum*. Several lineages having specific characteristics appear as result of environmental conditions and varieties of tobacco grown, adding to the considerable variation in morphology and physiology of the fungus. The close relationship of *F. oxysporum* isolates from cotton, tobacco and sweet potato can be linked to crop rotation, which is a common practice in some areas in the US (Clark et al., 1998). Recently, a new species of *Fusarium* was discovered, *Fusarium kyushuense* O'Donnell & T. Aoki. It was reported as the cause of *Fusarium* wilt in tobacco cv. Honghuadajinyuan in Guizhou, China (Wang et al., 2013). Even though *F. oxysporum* f. *nicotianae* is well accepted, there is discordance between fungi taxonomy databases as to whether to consider it as a *forma specialis* or not.

Wilt occurs in tobacco plants as a result of the presence and activity of the pathogen in the xylem. While the infected plant is alive, the fungus remains in the plant xylem, moving into other tissues to sporulate at or near to the surface once the tobacco plant dies (Agrios, 2004). The typical symptom of *Fusarium* wilt is the yellowing and drying of the leaves on one side of the plant. Wilting begins a few days after infection without being conspicuous, and young plants bleach to yellow or bronze and remain turgid for days. Due to unequal growth, the midribs of leaves are curved towards the infected side with half of the leaf yellowed and the other half remaining green (Reich, 1986).

Fusarium wilt is an example of a soil inhabitant, with capacity to survive more than ten years in the soil as chlamydospores. When environmental conditions are optimal and actively growing tobacco roots are present in the soil, the chlamydospores are able to germinate, because nutrients released to the rhizosphere create a suitable environment for fungus germination, growth and multiplication. Other factors such as temperature (disease is most severe between 28 and 31^0C) and soil moisture account for the severity of the disease. *Fusarium* wilt infection could be enhanced by the activity of tobacco cyst nematode and root-knot nematode (Shew and Lucas, 1991).

CHAPTER 3: Comparison of Methods for Generation of Doubled Haploids in Tobacco

3.1 Introduction

Production of doubled haploid lines (DH) is often used to significantly reduce the amount of time required to achieve homozygosity, because the use of classical inbreeding and selection techniques to obtain pure lines is relatively expensive and time consuming (Seguí-Simarro, 2010). Another advantage of this technique is the reduction in population size, since specific combinations of desirable genes can be found by screening a reduced number of DH individuals, compared to heterozygous populations (Chalyk, 2012).

The application of DH in tobacco breeding followed the successful establishment of an androgenic derived haploid (ADH) protocol in *Datura innoxia* Mill. in 1964, and was first used in burley tobacco in early 1970 (Kasperbauer and Collins, 1972). The ADH technique proved to be very effective and quickly became the primary method for DH production in tobacco. Generation of haploid plants from anthers is the result of distinct and complementary mechanisms differentially activated in the microspores, depending on the species and the nature of the environmental stress applied. Testillano et al. (2000) studying tobacco, pepper (*Capsicum annuum* L.) and *B. napus* demonstrated that heat stress triggers signal transduction of mitogen-activated protein kinase (MAPK), additionally to enrichment of heat shock protein (HSP70). By the time the use of the ADH method was well established, maternally derived haploids (MDH) obtained by interspecific hybridization between *N. tabacum* and *N. africana* (Burk et al., 1979) emerged as an alternative for haploid production in tobacco. The hybridization is followed by elimination of the paternal chromosomes, culminating in generation of haploid plants derived only from the female parent (Palmer et al., 2005).

ADH and MDH are currently the common methods used to originate DH lines in tobacco. Both methods have advantages and disadvantages with regard to techniques utilized, equipment and time required for their

production, as well as plant vigor and yield of the finished lines. Maternally derived doubled haploid (MDDH) and androgenic derived doubled haploid (ADDH) lines of flue-cured tobacco have been shown to be inferior to the cultivars from which they were derived when compared for overall agronomic performance. Although MDDH lines performed better than ADDH lines, neither displayed equal yielding ability as the selfed progenies of the parental line. The lower yields of ADDH in comparison to the parental lines could be due to somaclonal variation and inbreeding depression, where only nuclear DNA is involved in forming haploids (Wernsman et al., 1989). Other explanations for the unsatisfactory performance are the high levels of homozygosity (Niemirowicz-szczytt, 1997) and DNA amplification as consequence of the haploid regeneration process (Reed et al., 1994).

While the main complication of ADH is its association with the agronomic performance of the DH lines, for MDH the primary negative factor is the difficulty in the induction of haploids. Differential genotypic response in yield of haploids varying from 0 to almost 200% was observed in studies with several genotypes of onion (Bohanec, 2009). Burk et al. (1979) reported that the percentage of haploids among all surviving seedlings of three different flue-cured tobacco varieties hybridized with *N. africana* varied from 4.23 to 15.94%. Likewise, Nunes (2009) reported that burley tobacco produced one haploid per 2,100 seeds while for flue-cured this ratio was one haploid per 8,300 seeds, on average.

Unsatisfactory agronomic performance or difficulties in inducing haploids are not an exclusive problem in tobacco. In a comparison between modified single seed descent, conventional mass and DH breeding methods for maize inbred line development, the DH had the lowest proportion among the best lines for grain yield and yield:moisture ratio (Jumbo et al., 2011). In wheat, the simulation for the adaptation trait of genetic gain of a selected bulk selection method compared to DH indicated the former breeding strategy as genetically and economically more efficient (Li et al., 2013).

Successful improvements for production of tobacco DH have been made over the past decades, increasing the yield of ADDH lines. Even though on average ADDH lines do not perform satisfactorily as inbred populations, some lines within the DH population may equate and even surpass the yield

of inbred populations (Nielsen and Collins, 1989). Although several studies substantiate the inferiority of the ADH versus MDH method in the production of homozygous inbred lines or varieties, studies have not been conducted to clearly determine whether the differences are detectable when ADDH or MDDH lines are used only for the development of hybrid cultivars, which are typically now used for commercial tobacco production.

The main objectives of this study were: 1) to determine the most effective method to develop DH lines (ADH or MDH), based on relative ease of attaining haploid plants and the agronomic characteristics and yield of resultant DH lines; and 2) to determine whether any loss of vigor which may be detected in the DH lines, compared to the original parental lines, would carry over to their use in hybrid varieties, and if so, to what extent. The hypothesis was that any loss of vigor in the DH lines would likely be due to inbreeding depression which may result from the 100% homozygosity that would occur in a DH line; since hybrid cultivars are by definition heterozygous, loss of vigor in a DH line may be unimportant when used in hybrid combinations.

3.2. Materials and Methods

3.2.1 Generation of Doubled Haploid Populations

Two inbred burley tobacco lines were used to generate both ADDH and MDDH experimental lines. The purpose of using two inbred lines as a source of haploids was to detect any possible variation in the production of haploids, and agronomic performance of doubled haploids, that could be inherent to a specific genotype. In the literature, the number of tobacco haploid lines used to generate DH for comparison of agronomic traits with their respective inbred lines varied from one (Brown et al., 1981; Deaton et al., 1982), three (Oinuma and Yoshida, 1974; Kasperbauer et al., 1983), ten (Arcia et al., 1978; Schnell et al., 1980; Wernsman et al., 1989), twenty (Nielsen and Collins, 1989), twenty nine (Smalcelj et al., 2000), and up to thirty five (Deaton et al., 1986).

In the current study, the number of genetic sources used to produce DH lines was restricted to two based on limited resources available to carry

out field trials. The first source utilized was TN 90LC, a popular long-term inbred cultivar (circa sixteen generations of selfing) widely used for commercial production of burley tobacco. The second source utilized was inbred parental line GR 149LC (circa twelve generations of selfing). TN 90LC and GR 149LC were chosen for the project because extensive historical data were available not only for the inbred lines themselves, but also for hybrid varieties for which TN 90LC or GR149LC comprised one of the parental lines. TN 90LC is the male parent of hybrid cultivar KT 204LC, while GR 149LC is the male parent of hybrid cultivar TN 97LC. The use of TN 90LC and GR 149LC as the source materials therefore enabled a direct comparison not only between ADDH and MDDH lines within each cultivar itself, but also allowed a direct comparison between the ADDH and MDDH lines when used to produce hybrid varieties.

3.2.1.1 Induction of Androgenic Derived Haploids

For TN 90LC, a diversity of plants including field grown, young and old greenhouse plants, rooted apical shoots, and plants treated with a 20X etridiazole (to initiate early flowering) were used to produce ADH plants. The objective of using multiple sources of diploid plants was to determine whether any of the plant sources evaluated would be unsuitable for ADH production due to unsatisfactory number of haploids generated. Since a second objective was to determine the shortest time possible for the production of haploids, the etridiazole treatment was included for TN 90LC. Etridiazole, which is the active ingredient of the fungicide Terramaster® 4EC, is labelled for use in tobacco to prevent and/or cure *Pythium* root rot in tobacco seedbeds. etridiazole belongs to the group of triazols, which are known for its inhibitory effect on plant growth when mismanaged. An excessive rate of Etridiazole inhibits biosynthesis of the hormone gibberellin, which is responsible primarily for shoot and stem elongation, resulting in early flowering of tobacco plants. Since the objective of this project was to increase efficiency in producing haploids, etridiazole was used at twenty times the recommended concentration in greenhouse seedbeds to force early flowering of plants, shortening the time from germination until anthesis. For each source of TN

90LC plants utilized in the preliminary studies, the number of diploid plants used, number of flower buds harvested, and number of anthers plated are listed in Table 3.1. For GR 149LC, only field grown plants were utilized for the generation of ADH plants.

For the collection of anthers, immature flower buds were harvested when the corolla was visible inside the sepals and about the same length as the calyx. The immature flowers were wrapped in a paper towel and aluminum foil, with the paper having the function of absorbing excessive moisture and the aluminum foil to prevent the light from reaching the buds. The immature flowers were kept at 4^{o}C for seven days; on the seventh day the anthers were extracted after disinfestation of the flower buds. To disinfest, the flower calyx was removed to allow maximum exposure of the corolla. The buds were washed in 70% ethanol (v/v) for 30 s (seconds), followed by immersion in 2.63% sodium hypochlorite solution for 2 min (minutes), followed by three rinses with sterile distilled water.

Extracted anthers were plated on A-medium (anther culture medium) (Kasperbauer and Wilson, 1979) in Petri plates (100 mm x 15 mm; 30 ml medium). Petri plates were taped with Parafilm and incubated for seven days at 23 ± 1^{o}C in a dark chamber, then placed in a growth chamber at 28 ± 1^{o}C with a 16-hour photoperiod provided by white fluorescent light bulbs (Light intensity of approximately 150 µmol m^{-2} s^{-1}). Approximately three weeks later, actively growing shoots containing at least two leaf primordia were excised from anthers and transplanted onto Murashige-Skoog (MS) rooting medium (Murashige and Skoog, 1962), supplemented with 2 ml l^{-1} of Plant Preservative Mixture™ (PPM) (Plant Cell Technology, Inc., Washington, DC). The induction of roots of the explanted shoots in the MS medium was performed using 20 x 100 mm culture tubes (15 ml medium)**.**

Table 3.1 – Androgenic derived haploid procedure

Genotype	Plant type / Environment	Inbred Plants (No)	Flower buds (No)	Anthers plated (No)	Anthers germinated (No)	Haploid explants (No)
TN 90LC	Young / Field	3	32	155	3	47
	Terramaster / GH	10	25	115	0	0
	Sucker growth / GH	2	10	49	0	0
	Old plant / GH	2	10	49	0	0
	Young plant / GH	2	10	50	0	0
GR 149LC	Young / Field	2	62	272	39	35

The ploidy level was verified when haploid candidates had a robust root system and leaves in expansion, using a Partec PA-1 flow cytometer with mercury arc lamp (Partec North America, Inc., Swedesboro, NJ). Leaf DNA was extracted in a Petri dish by adding 400 µl of CyStain UV precise P nuclei extraction buffer, followed by chopping the tissue using a razor blade. After at least 30 seconds of incubation, 1.6 ml of CyStain UV precise P Staining buffer was added and the material was filtered through a Partec 50 µm CellTrics disposable filter. A total of 47 ADH plants were verified for TN 90LC, while 35 ADH plants were verified for GR 149LC (table 3.1). For both varieties, 10 plantlets were randomly selected and transferred into 1.5 L capacity plastic pots and grown in the greenhouse under artificial light supplied by 1000 watts mercury lamps placed 1.8 m above the plants.

3.2.1.2 Induction of Maternally Derived Haploids

For the MDH method, the plant source, number of plants used, and the number of *N. africana* interspecific crosses for both TN 90LC and GR 149LC are presented in Table 3.2. Plants of TN 90LC and GR 149LC were grown in the field and used for interspecific crosses in 2011 and 2012, respectively. A total of 32 field crosses were made for TN 90LC on August 26, 2011 (29.4^{o}C). A total of 117 pollinations were made for GR 149LC on July 21 (27.2^{o}C), July 25 (35.5^{o}C), and August 1, 2012 (32.2^{o}C). Plants of TN 90LC and GR 149LC were also grown in a greenhouse in 1.5 L plastic pots using Peat-Lite Tobacco Mix (Carolina Soil Company, Kinston, NC). The female plants were situated 60 cm below 1000 watts mercury lamps at a temperature of 30 ± 5^{o}C. For both the greenhouse and field crosses, immature, unopened flowers were emasculated to avoid self-pollination, then pollinated with pollen of *N. africana*. A total of 82 and 343 pollinations with *N. africana* were made in the greenhouse for TN 90LC and GR 149LC, respectively (table 3.2).

Table 3.2 – Maternally derived haploid procedure

Genotype	Plant type / Environment	Inbred Plants (No)	Interspecific Crosses (No)	Haploid Candidates (No)	Haploid plants (No)	Crosses/ haploid ratio
TN 90LC	Young / Field	3	32	22	0	0
	Terramaster / GH	24	36	23	0	0
	Sucker growth / GH	2	15	13	5	3
	Old plant / GH	2	15	15	3	5
	Young plant / GH	2	16	15	2	8
GR 149LC	Young / Field	2	117	-	2	88.5
	Young plant / GH	2	343	-	2	171.5

Between 25 and 35 days after pollination, the seed capsules were harvested, dried with 40^{o}C air flow for one day, and progeny seeded in individual plastic trays using PRO-MIX® BX growing medium (Premier Tech Horticulture, Quebec, Canada). As expected from an interspecific incompatible cross, the vast majority of the seedlings perished just after germination; the plants that survived were morphologically separated into haploid and non-haploid candidates. The ploidy level was later confirmed by flow cytometry analysis using the same equipment and procedures used for ADH.

For TN 90LC, ten MDH plants were verified and randomly assigned numbers 1-10. However, although 460 GR 149LC flowers were cross pollinated with *N. africana* in either the field or the greenhouse, only four MDH GR 149 plants were identified; these plants were randomly assigned numbers 1-4. The MDH plants from both TN 90LC and GR 149LC were transferred into 1.5 L capacity plastic pots and grown in a greenhouse under artificial light supplied by 1000 watt mercury lamps placed 1.8 m above the plants.

3.2.1.3 Chromosome Doubling to Create ADDH and MDDH Lines

The induction of chromosome doubling for both ADH and MDH lines was done by culturing tissues of the leaf petiole. Actively growing leaves were collected from haploid plants when the base of the leaf was at least 10 mm (millimeters) wide. The 50 mm basal part of the petiole was used for culture. The explants were surface sterilized by immersion in 70% ethanol for 45 s, followed by incubation in 1.05% sodium hypochlorite solution for 5 min, followed by a final rinse with sterile distilled water. A few mm of each end of the tissue were discarded and the remaining 30 to 40 mm long tissue was cultured horizontally on MS shoot induction medium for adventitious shoot initiation.

The shoot induction medium was composed of regular MS medium supplemented with 2 ml $l^{-1}$of PPM, 4 mg $l^{-1}$of IAA (Indole Acetic Acid), and 2.5 mg l^{-1} of Kinetin. Magenta GA-7 plant culture vessels (Magenta Corp., Chicago, IL) containing 40 ml of medium were used to culture the petiole tissues. Apical shoots that formed were extracted and rooted on MS medium.

Once plant growth was established, a leaf tissue sample was analyzed for doubled haploidy. DH candidates were tested using Partec PA-1 flow cytometer with mercury arc lamp, and the verified DH plants were transferred to 1.5 L capacity plastic vessels and grown in the greenhouse until seed capsules were mature.

For TN 90LC, a total of ten ADDH and ten MDDH lines were produced from unique ADH and MDH haploid sources. The ten ADDH TN 90LC were designated as TN 90LC ADDH1 through TN 90LC ADDH10, and the ten MDDH TN 90LC lines were designated as TN 90LC MDDH1 through TN 90LC MDDH10. In the case of GR 149LC, ten ADDH lines were produced from unique ADH plants; these lines were randomly designated as GR 149LC ADDH1 through GR 149LC ADDH10. However, only four MDH GR 149LC plants were identified and available for the production of MDDH lines; the resultant MDDH lines were randomly designated as GR 149LC MDDH1 through GR 149LC MDDH4.

3.2.1.4 Hybrids Derived from Doubled Haploid Populations

The second objective of the comparison of methods for the generation of doubled haploids in tobacco was to determine how the MDDH and ADDH lines performed as parental lines when used to create hybrid cultivars. For TN 90LC, this was accomplished by utilizing the commercial cultivar KT 204LC, which is a hybrid cross between maternal parent TKS 2002LC and paternal parent TN 90LC. Pollen was collected from each of the ten ADDH and ten MDDH lines of TN 90LC and crossed onto TKS 2002LC to produce twenty hybrid lines. These KT 204LC lines were numbered in the same manner used for the parental line study. For example, the cross TKS 2002LC x TN 90LC ADDH1 was designated KT 204LC AD(F_1)1; the cross TKS 2002LC X TN 90LC ADDH5 was designated as KT 204LC AD(F_1)5; the cross TKS 2002LC X TN 90LC MDDH7 was designated as KT 204LC MD(F_1)7, etc.

The commercial cultivar TN 97LC was utilized to compare the relative performance of GR 149LC ADDH and MDDH lines when used to create hybrid cultivars. TN 97LC is a hybrid cross between maternal parent ms TN 90LC and paternal parent GR 149LC. Pollen was collected from each of the

ten ADDH and four MDDH lines of GR 149LC and crossed onto ms TN 90LC to produce fourteen hybrid lines. The ten TN 97LC hybrid lines having ADDH lines as a pollinator were designated as TN 97LC AD(F_1)1 through TN 97LC AD(F_1)10, while the four hybrids having MDDH lines as the pollinator were designated as TN 97LC MD(F_1)1 through TN 97LC MD(F_1)4.

3.2.2 Field Trials and Evaluations

To allow direct comparisons among ADDH, MDDH, and original inbred parental lines in the field trials, TN 90LCAD1 and TN 90LCMD1 were paired with inbred line TN 90LC to form a triplet designated as No1; TN 90LCAD2 and TN 90LCMD2 were paired with TN 90LC to form a second triplet designated as No2 and so on, with the last triplet designated as No10 (table 3.3).

In the case of GR 149LC doubled haploids, there were ten ADDH derived hybrids but only four MDDH derived hybrids available for comparison with the original GR 149LC. This limited number of GR 149LCMD lines created a problem in comparing ADDH and MDDH lines with the original GR 149LC source. To maintain a balanced experimental field design, the four GR 149LCMD lines were used twice in the field trials, while eight of the ten GR 149LCAD lines were used once. GR 149LCAD1 through GR 149LCAD4 and GR 149LCMD1 through GR 149LCMD4 were paired with GR 149LC to form triplets No1 through No4, while GR 149LCAD5 through GR 149LCAD8 and GR 149LCMD1 through GR 149LCMD4 were paired with GR 149LC to form triplets No5 through No8 (table 3.3). This distribution was necessary to balance the frequency of lines within the main plots of the split-plot design. Similar pairings were made within the field trials evaluating KT 204LC and TN 97LC hybrid lines (table 3.4).

The field trials were conducted at four locations: the UK Spindletop Research Farm near Lexington, KY (LX); the UK C. Oran Little Research Farm near Versailles, Woodford County, KY (WC); the University of Tennessee Agricultural Experiment Station near Greeneville, TN (GR); and the Highland Rim Research and Educational Center near Springfield, TN (HR).

Table 3.3 - Split-plot design used for the TN 90LC and GR 149LC trials.

Main	------- TN 90LC subplot -------			------- GR 149LC subplot -------		
Plot	ADDH	inbred	MDDH	ADDH	inbred	MDDH
1	No 1	No 1	No 1	No 1	No 1	No 1
2	No 2	No 2	No 2	No 2	No 2	No 2
3	No 3	No 3	No 3	No 3	No 3	No 3
4	No 4	No 4	No 4	No 4	No 4	No 4
5	No 5	No 5	No 5	No 5	No 5	No 1
6	No 6	No 6	No 6	No 6	No 6	No 2
7	No 7	No 7	No 7	No 7	No 7	No 3
8	No 8	No 8	No 8	No 8	No 8	No 4
9	No 9	No 9	No 9	-	-	-
10	No 10	No 10	No 10	-	-	-

Table 3.4 - Split-plot design used for the hybrids KT 204LC and TN 97LC trials.

Main Plot	------- KT 204LC subplot -------			--------- TN 97LC subplot --------		
	AD(F_1)	hybrid	MD(F_1)	AD(F_1)	hybrid	MD(F_1)
1	No 1	No 1	No 1	No 1	No 1	No 1
2	No 2	No 2	No 2	No 2	No 2	No 2
3	No 3	No 3	No 3	No 3	No 3	No 3
4	No 4	No 4	No 4	No 4	No 4	No 4
5	No 5	No 5	No 5	No 5	No 5	No 1
6	No 6	No 6	No 6	No 6	No 6	No 2
7	No 7	No 7	No 7	No 7	No 7	No 3
8	No 8	No 8	No 8	No 8	No 8	No 4
9	No 9	No 9	No 9	-	-	-
10	No 10	No 10	No 10	-	-	-

The soil type and classification were Bluegrass-Maury silt loam (fine, mixed mesic typic Paleudalf) for the LX and WC location; Cumberland silt loam [undulating phase (dewey)] for GR; and Dickson silt loam (with 5% Sango) for HR.

The ADDH lines, MDDH lines, and the inbred parental lines were transplanted as a split-plot design with three replications. Main plots consisted of lines, comprising one ADDH, one MDDH and one inbred line (table 3.3) and the sub-plots consisted of methods. Individual plots consisted of 32 plants spaced 0.53 m between plants (0.41 m at GR) and 1.07 m between rows. Data were collected for plant height at the 50th day after transplanting (height 50), plant height after topping (height topping), leaf length, leaf width, number of leaves per plant, and yield/hectare. Agronomic traits were estimated based on six individual plants per plot; yield/hectare was estimated from the entire 30 plant plot (the two end plants of each plot were discarded) after harvest and curing. Data for plant height at the 50th day after transplanting were collected by measuring the length of the plant between the soil line and the apical meristem. The assessment of height after topping was performed by measuring the stalk height from the soil line to the leaf axil of the top leaf. The distance between the petiole and the leaf tip, and the distance between the borders at the widest point on the sixth leaf from the top of the plant, were used to estimate leaf length and leaf width, respectively. The number of leaves per plant was recorded between the second and fourth week after the crop was topped.

The field trials of the TN 90LC lines were conducted during the 2013 growing season at four locations (LX, WC, GR, and HR). Yield data were collected at all four locations, but agronomic data were not collected at the HR location. The field trials for the GR 149LC, KT 204LC, and TN 97LC lines were conducted during the 2014 growing season at three locations - WC, GR and HR (the LX location was transplanted but was not usable due to severe hail damage). Transplant, topping and harvest dates are presented in Table 3.5. Data collected were as described for the TN 90LC DH trials.

3.2.3 Data Analysis

Combined analyses of variance (including all locations) for each one of the genotypes were performed for all six traits measured (height 50, height topping, leaf length, leaf width, number of leaves per plant and yield/hectare). The four genotypes (TN 90LC, KT 204LC, GR 149LC and TN 97LC) were analyzed separately using the General Linear Model (GLM) procedure (SAS version 9.3, 2014). The TN 90LC ADDH5 and TN 90LC MDDH5 lines were discarded from the analyses before performing ANOVA, because those two lines represented outlier data points.

Location, line, method and the interaction effects were evaluated as a split-plot analysis using the following model:

$$Y_{ijkm} = \mu + L_i + G_k + R_{j(i)} + [GR_{kj(i)}] + M_m + [MG_{mk}] + [ML_{mi}] + [MGL_{mki}] + E_{ijkm}$$

Where: Y_{ijkm} = the observation of the j^{th} replication , k^{th} genotype and m^{th} method at the i^{th} location,

μ = overall mean,

L_i = the effect of the i^{th} location,

G_k = the effect of the k^{th} genotype,

$R_{j(i)}$ = the effect of j^{th} replication nested within i^{th} location,

$GR_{kj(i)}$ = the effect of the interaction of k^{th} genotype and j^{th} replication nested within i^{th} location,

M_m = effect of the m^{th} method,

MG_{mk} = the effect of the interaction of m^{th} method and k^{th} genotype,

ML_{mi} = the effect of the interaction of m^{th} method and i^{th} location,

MGL_{mki} = the effect of the interaction of m^{th} method and k^{th} genotype in the i^{th} location

E_{ijkm} = the residual error

Note: Hypothesis testing for L_i considered $R_{j(i)}$ as the error term and hypothesis test for G_k had $GR_{kj(i)}$ effect as its error term.

Means separation – Individual lines within each method

For each of the TN 90LC, GR 149LC, KT 204LC, and TN 97LC families, the lines within every population were analyzed to estimate differences between means. Analysis of variance was used to detect differences between lines within each population, using the following model for all six traits measured:

$$Y_{ijkm} = \mu + L_i + R_{j(i)} + G_k + G_{k(i)} + E_{ijkm}$$

Where: μ = overall mean,

L_i = the effect of the i^{th} location,

$R_{j(i)}$ = the effect of j^{th} replication nested within i^{th} location,

G_k = the effect of the k^{th} genotype,

$G_{k(i)}$ = the effect of k^{th} genotype nested within i^{th} location,

E_{ijk} = the residual error

In the populations in which significance was detected, Fischer's Least Significant Difference (LSD) was used to separate means of lines.

Table 3.5 - Transplant, topping and harvest dates of TN 90LC, GR 149LC, KT 204LC and TN 97LC trials.

Location	Transplant	Topping	Harvest
---------- TN 90LC Populations ----------			
LX	May 24 2013	July 26 2013	Aug 23 2013
WC	May 30 2013	July 23 2013	Aug 28 2013
GR	May 30 2013	July 26 2013	Aug 29 2013
HR	May 30 2013	Aug 07 2013	Sep 06 2013
---------- GR 149LC Populations ----------			
WC	May 30 2014	Aug 13 2014	Sep 22 2014
GR	May 27 2014	July 31 2014	Sep 03 2014
HR	May 20 2014	Aug 13 2014	Sep 09 2014
---------- KT 204LC Populations ----------			
WC	May 30 2014	Aug 06 2014	Sep 17 2014
GR	May 27 2014	July 31 2014	Sep 02 2014
HR	May 20 2014	Aug 05 2014	Sep 05 2014
---------- TN 97LC Populations ----------			
WC	May 30 2014	Aug 06 2014	Sep 10 2014
GR	May 27 2014	July 30 2014	Sep 03 1014
HR	May 20 2014	Aug 07 2014	Sep 09 2014

Means separation – Methods within each line (triplet)

For each of the TN 90LC, GR 149LC, KT 204LC, and TN 97LC families, the methods within the lines (triplet) of every population were analyzed to estimate differences between the means of the three methods. The analysis of variance was performed based on lines, as randomized complete block using the General Linear Model (GLM) procedure (SAS version 9.3, 2014), following the model:

$$Y_{ijm} = \mu + L_i + R_{j(i)} + M_m + ML_{mi} + E_{ijm}$$

Where: μ = overall mean,
L_i = the effect of the i^{th} location,
$R_{j(i)}$ = the effect of j^{th} replication nested within i^{th} location,
M_m = effect of the m^{th} method,
ML_{mi} = the effect of the interaction of m^{th} method and i^{th} location,
E_{ijm} = the residual error

In all triplets (methods within lines), Fischer's Least Significant Difference (LSD) was used to separate means.

3.3 Results and discussion

3.3.1 Efficiency of Obtaining Haploids

Androgenic Derived Haploids

One of the primary objectives of the current research was to determine the relative efficiency of producing ADH and MDH plants. For TN 90LC, plants grown under a wide range of specific conditions were utilized to see if they could be successfully utilized to generate haploid plants. The efficiency in inducing ADH in the TN 90LC burley variety was dependent on the environment in which donor plants were grown (table 3.1). Anthers collected from plants grown under field conditions successfully produced haploid plants, but none of the 263 anthers originating from TN 90LC plants cultivated under

different conditions in the greenhouse generated haploid plants. Using young field grown plants, 155 anthers of inbred TN 90LC were used to generate ADH plants; even though only three (1.9%) of the anthers germinated satisfactorily, they yielded 47 haploid plants (table 3.1). From just one of those three anthers, 36 haploid plants were extracted and successfully used to produce DH lines.

For GR 149LC, only field grown plants were utilized; they also produced a satisfactory number of haploid plants. From young field grown plants of GR 149LC, 39 of 272 (14.3%) of the plated anthers germinated; these were used as a source of at least one haploid plant per anther. Some anthers of GR 149LC produced more than ten haploid plants (data not shown), but since the objective of this project was to obtain a population of ten ADH from each genotype, data was not collected beyond the number of ADH lines required.

The inability to produce any ADH plants from TN 90LC plants grown in the greenhouse, including young plants that were equivalent to those that were successfully utilized to produce ADH plants in the field, was surprising. A difference in photoperiod at certain light intensity can induce a five-fold difference in the yield of haploids (Dunwell and Sunderland, 1974). The inability to derive ADH plants from rooted axillary shoots, or from plants where Terramaster® 4EC was used to initiate early flowering, was particularly disappointing. One of the primary reasons for using DH procedures in a tobacco breeding program is to shorten the breeding process. If DH procedures could be coupled with other techniques used to shorten breeding cycles, the overall time required to obtain homozygous inbred lines could be reduced even further. The effect of the vigor of the plant from which anthers are harvested on haploid production has been reported in tobacco. Kasperbauer and Collins (1974) noted that changes in growth conditions of donor plants cause floral buds to abort. They considered the selection of vigorous and healthy floral buds to be the most important environmental factor in determining the success of haploid generation. Robust and healthy plants produce larger anthers, which are more vigorous and less likely to senesce due to stresses during culture, resulting in improved germination of haploid cells.

Maternally Derived Haploids

Based on the results observed in TN 90LC, the type of plant used for pollination with *N. africana* also influenced the generation of haploid plants, but in a converse manner than was observed for the production of ADH plants (table 3.2). From plants grown in the greenhouse, rooted axillary shoots and young and old TN 90LC plants were able to generate haploids. From these sources, 46 flowers of TN 90LC plants were crossed with *N. africana* to produce ten MDH lines. Although 36 crosses were made using plants grown with Terramaster, no haploids plants were obtained. There were also no haploid plants obtained from the 32 interspecific hybridizations performed in the field. Several factors could be involved in the lack of haploid production, but the fact that those combinations of plant types and environments had the highest number of crosses but yielded no TN 90LC haploids should be considered when inducing maternally derived haploids. The average number of *N. africana* crosses necessary to generate one haploid plant of TN 90LC was 11.4 for all sources of female plants; considering just the sources of plants which produced haploids, the ratio was 4.6/1.

For GR 149LC, a very low incidence of MDH plants were identified, regardless of whether the interspecific crosses were made in the greenhouse or in the field (table 3.2). In the field, 117 interspecific crosses produced only two MDH plants, while in the greenhouse 343 crosses produced only two haploid plants. The ratio of the number of crosses/number of haploids for TN 90LC and GR 149LC was 9.4/1 and 115/1, respectively. This lack of MDH production for GR 149LC compared to TN 90LC suggests that there may be a significant cultivar effect on MDH production in burley tobacco.

Under greenhouse conditions, Burk et al. (1979) reported that tobacco plants of burley variety “VT-9“ and flue-cured cultivar “NC 95” pollinated with *N. africana* produced an average of 2800 seeds per capsule and the germination varied from 94 to 100%. Most of the seedlings died before developing true leaves, with just 0.25 to 1.42% of the germinated individuals surviving. In the cross with VT-9, 4.23% of the germinated plants were haploids, while in the hybridization with the flue-cured line, 15.94% of the germinated progeny was haploid. The F_1 interspecific progeny of the burley

and the flue-cured cultivars yielded an average of 1 and 3.7 haploids per cross (seed capsule), respectively. In southern Brazil, one flue-cured line and one burley line were crossed with *N. africana* to produce MDH plants (Nunes, 2009). Approximately one burley haploid plant was derived from every cross with *N. africana*, but almost 3 crosses were needed to generate one haploid in flue-cured tobacco. The ratios of haploid/ number of seeds seeded were 1/8,300 for flue-cured and 1/2,100 for burley.

The Burk and Nunes studies above stated that roughly one haploid will be generated from each interspecific cross (seed capsule). The data obtained from both TN 90LC and GR 149LC differed significantly from those previous findings. Around ten interspecific crosses were necessary to produce one MDH haploid of TN 90LC and more surprisingly, 115 crosses with *N. africana* were necessary to yield one haploid individual in the GR 149LC genotype. The data presented in Table 3.2 show drastic differences between the two burley inbred lines with regard to the ability to generate haploid plants. Differential response in the number of haploids generated in each interspecific cross can also be noted by analyzing the results of the Burk and Nunes studies. The flue-cured variety NC 95 yielded an average of one haploid per 760 seeds, when crossed with *N. africana* (Burk et al., 1979). But the results of Brazil study reported 8,300 seeds were necessary to produce one haploid in flue-cured tobacco (Nunes, 2009). This was a difference of almost eleven fold between the two studies.

Anthers of both TN 90LC and GR 149LC were able to generate a good number of haploids, but the efficiency of the MDH method in producing large numbers of DH was low, especially for GR 149LC. The MDH method was inferior to the ADH technique in this project and also much less efficient than the MDH method in other tobacco varieties from past studies.

3.3.2 Agronomic Performance of Doubled Haploid Lines - Results

TN 90LC family – Location effects

The analysis of variance (ANOVA) for agronomic traits for the TN 90LC family across locations is presented in Table 3.6, with the mean data

presented in Table 3.7. For all six traits measured, there were statistically significant differences. Among the three locations where field data were collected, the WC location ranked first for all traits, except number of leaves, for all three TN 90LC populations (table 3.7). Conversely, the LX location ranked last for all traits except plant height at topping and leaf number, which are the two variables that are somewhat subjective since topping height by differing labor crews is typically quite variable from location to location. Yield was also highest at the WC location, followed by GR, HR and LX. Environmental factors contributed to the differences among locations. The LX location recorded excessive rainfall in 2013, especially at the beginning of the vegetative phase in the field; insufficient drainage for the area in which the trial was located resulted in standing water and compounded the adverse effects on plant growth. The GR location received excessive rain fall in 2013, but the field where the trial was located was on a slope that provided better drainage than the LX site. The WC trial was set one week later than Lexington and was located at the top of a field that also had a considerable slope, which contributed to rapid water runoff and absence of standing water.

TN 90LC family – Comparison of methods for obtaining DH lines

The ANOVA for differences among methods of generating haploid lines of TN 90LC is presented in Table 3.8. The ANOVA revealed that on average over the three locations, 50 days after transplanting the plant height of both the ADDH and MDDH TN 90LC populations were significantly different from the inbred TN 90LC. The MDDH TN 90LC population was 4.4 cm (3.8%) taller than the inbred TN 90LC check, but the ADDH population was 6.7 cm (5.7%) shorter than the check (table 3.9). This trend was consistent at all three locations (table 3.7).

Table 3.6 - Analysis of variance of differences among locations for the TN 90LC trial.

Statistic	Height 50	Height topping	Leaf length	Leaf width	Leaf Number	Yield
DF	2	2	2	2	2	3
MS	56463.15	7451.00	2074.96	1263.16	19.23	5812130.25
F value	714.86***	115.09***	293.02***	541.39***	18.82***	57.51***

(*), (**), (***) - Differences significant at P<0.05, 0.01, 0.0001, respectively.

Table 3.7 – Agronomic traits means for the TN 90LC family by locations.

Trait	Location	Methods		
		ADDH	Inbred	MDDH
Height 50 (cm)	GR	100.2	109.7	114.1
	LX	91.9	98.3	98.6
	WC	141.0	145.2	153.7
Height topping (cm)	GR	123.3	129.5	131.8
	LX	138.0	142.5	144.7
	WC	146.6	146.0	148.9
Leaf length (cm)	GR	59.2	60.6	61.1
	LX	54.2	53.5	52.5
	WC	63.8	65.4	64.9
Leaf width (cm)	GR	26.0	27.1	27.0
	LX	21.2	22.1	21.1
	WC	29.1	31.1	30.4
Number of leaves per plant (No)	GR	19.1	19.3	19.6
	LX	20.5	20.3	20.5
	WC	20.2	19.8	20.0
Yield (Kg/ha)	GR	2811	3073	3090
	LX	2631	2604	2590
	WC	3078	3159	3255
	HR	2587	2720	2847

Table 3.8 - Analysis of variance of differences among TN 90LC methods (source of variation).

Comparison	Statistic	Height 50	Height topping	Leaf length	Leaf width	Leaf Number	Yield
Between All three populations	DF	2	2	2	2	2	2
	MS	2543.43	589.67	11.16	30.8	0.96	806231
	F value	32.2***	9.11**	1.58	13.2***	0.94	7.98**
Inbred x ADDH	DF	1	1	1	1	1	1
	MS	1816.91	394.82	22.01	61.5	0.42	600509
	F value	26.03***	5.49*	3.42	31.35***	0.33	5.28*
Inbred x MDDH	DF	1	1	1	1	1	1
	MS	795.78	205.11	3.48	13.22	1.91	170509
	F value	10.96**	4.86*	0.46	5.04*	3.19	1.84

(*), (**), (***) - Differences between populations significant at P<0.05, 0.01, 0.0001, respectively.

The ADDH lines grew more slowly than the inbred source, as measured by the plant height at the 50th day after transplant. The results are in accord with the findings in the flue-cured tobacco varieties NC 95 and Coker 139 by Arcia et al. (1978). The same trend was not observed in oriental tobacco varieties; in a comparison between ADDH lines and inbred sources of three oriental tobacco varieties, Miceska (2009) reported that the ADDH method was taller compared to the inbred populations in two of the varieties tested, while one inbred population of oriental tobacco was taller than the ADDH lines derived from it.

The trend observed for plant height at the 50th day after transplanting persisted for plant height after topping; both the ADDH and MDDH populations were significantly different from the inbred TN 90LC (table 3.8). On average, the tallest population after topping was MDDH TN 90LC (141.4cm), followed by the inbred check TN 90LC (138.9cm) and the ADDH TN 90LC population (135.7cm) (table 3.9). The MDDH TN 90LC population was 1.8% taller than the inbred TN 90LC population; this difference was statistically significant. The ADDH TN 90LC population was 2.3% shorter than the inbred check TN 90LC and also significantly different (table 3.8). The mean plant height at topping for the different methods at the locations was similar to plant height 50 days after transplant; the ADDH population was the shortest and the MDDH population was the tallest at all locations (table 3.7).

Previous research also found the ADDH technique inferior to conventional strategies to produce highly homozygous burley lines. The average plant height of ADDH lines of five burley inbreds was smaller than the average height of the five inbred sources (Kasperbauer et al., 1983). Statistically significant differences for plant height after topping were also reported among ADDH lines of both populations of “NC 95” and “Coker 139” flue cured cultivars (Arcia et al., 1978). In another study, Schnell et al. (1980) reported that ADDH lines derived from F_1 line of the cross between two flue-cured varieties (Hicks Broadleaf x Coker 139) were less vigorous than the lines inbred using SSD method and also inferior to both parental lines.

Table 3.9 - Mean, standard deviation, standard error, coefficient of variation, minimum and maximum values for the inbred TN 90LC, ADDH and MDDH populations over three locations - 2013 (GR, LX, WC).

Statistics	Height 50 (cm)	Height topping (cm)	Leaf length (cm)	Leaf width (cm)	Leaves per plant No)	Yield (Kg/ha)
---------- ADDH TN 90LC population ----------						
Mean	111.02	135.72	59.64	25.97	19.84	2778
Std Dev	26.69	15.85	4.74	3.67	1.29	361.44
Std Error	2.97	1.87	0.56	0.43	0.15	35.27
CV	0.24	0.12	0.08	0.14	0.07	0.13
Min	77.86	104.25	47.2	17.3	16.38	1673
Max	176.6	167.13	68	33.13	22	3619
---------- Inbred TN 90LC population ----------						
Mean	117.71	138.94	60.63	27.35	19.73	2889
Std Dev	26.75	13.40	5.55	3.98	1.10	409.98
Std Error	2.82	1.50	0.62	0.44	0.12	37.43
CV	0.23	0.1	0.09	0.15	0.06	0.14
Min	83.3	115.75	47.9	17.8	16.63	1931
Max	184.3	166.38	70.69	35.56	21.63	3777
---------- MDDH TN 90LC population ----------						
Mean	122.15	141.41	60.39	26.78	19.97	2945
Std Dev	29.08	13.8	5.87	4.16	1.21	444.03
Std Error	3.23	1.63	0.69	0.49	0.14	42.73
CV	0.24	0.1	0.1	0.16	0.06	0.15
Min	78.86	107	46.8	17.8	16.25	1647
Max	193	172.5	72.38	33.94	21.88	3938

It is important to note that even though the plant height of the TN 90LC ADDH population was, on average, shorter than the inbred source population, some lines within the ADDH population were taller than some lines in the inbred TN 90LC population. The differences will be discussed in more detail in the section “Comparison of individual lines within methods for obtaining DH lines”.

The observations of this research contradict previous DH studies in burley tobacco. Deaton et al. (1982) showed that the plant heights at topping of ADDH lines of seven burley cultivars were not significant different from their respective source cultivars. The performances of the ADDH lines were distributed in both directions around the average plant height of their source cultivars. Nielsen and Collins, (1989) observed that three out of the four five-lines sets of KY 17 ADDH and KY 17 MDDH were not significantly different from inbred source lines and one set of each method was actually inferior to the inbred population. In another study comparing 50 conventionally inbred and 35 ADDH lines of burley variety KY 16, Deaton et al. (1986b), reported no significantly differences in plant height among the two methods. Vigor reduction was detected in some DH lines (but the overall mean was not different from the inbred population) and the DH population displayed higher variability than the conventionally inbred.

The average length of the leaves of the inbred TN 90LC population was 60.6 cm, which was 1 cm and 0.22 cm longer than the ADDH and MDDH TN 90LC populations, respectively (table 3.7). These numbers represents 1.6% difference between the average leaf length of the inbred check TN 90LC and ADDH populations and 0.4% difference between inbred and MDDH TN 90LC populations. None of these differences in leaf length were statistically significant (table 3.8). When comparing ADDH lines of seven different burley inbred cultivars, Deaton et al. (1982) also did not find differences in leaf length between ADDH lines and the parental cultivars. In flue-cured, Smalcelj et al. (2000) reported no significant differences in a comparison of a population of 47 ADDH lines of flue-cured tobacco “DH 10” with the hybrid source. Wernsman et al. (1989) also reported no differences in leaf length between MDDH lines and inbred populations of KY 17, KY 10 and KY 15, but the

research did report the inferiority of the ADDH method in all three burley varieties.

In a comparison between 50 lines of KY 16 burley variety and 35 ADDH lines of the same genotype, Deaton et al. (1986a) reported significant differences among the methods, with the DH population producing shorter leaves and exhibiting greater variation among the lines. The ADDH and MDDH populations of TN 90LC in this study displayed significant differences between lines (within the same population), but not between populations. Similar behavior was observed by the majority of the previous studies, where the performance of the DH lines varied considerably around the average leaf length of the inbred parental sources.

For leaf width**,** both ADDH and MDDH populations were significantly different from the inbred check TN 90LC population (table 3.8). The inbred check TN 90LC displayed an average leaf width of 27.4cm, which was 5% wider than the ADDH TN 90LC population that had an average leaf width of 26 cm (table 3.9). TN 90LC was also superior to the MDDH TN 90LC population; the MDDH population had an average leaf width of 26.8cm, which was 2.1% less than the check TN 90LC check population (table 3.9). Kasperbauer et al. (1983) showed differential response of populations of ADDH lines derived from two different inbred lines. While ADDH lines of burley variety KY 16 displayed narrower leaves than the inbred source, the ADDH lines of Judy's Pride variety produced wider leaves compared to the inbred source. Several previous studies did not detect statistically significant differences for leaf width between ADDH and inbred source populations of burley tobacco. For example, Deaton et al. (1986a) compared 35 ADDH and 50 inbreds lines of KY 16; Wernsman et al. (1989) compared populations of varieties KY 10, KY 15, and KY 17; and Nielsen and Collins, (1989), compared ADDH lines of KY 17 with the inbred source. None of these studies showed significant differences between ADDH lines and the inbred sources for leaf width.

The average number of leaves per plant was 19.8, 19.7, and 20 for the ADDH, inbred, and MDDH TN 90LC populations, respectively (table 3.9). There were no statistically significant differences between any of the methods compared (table 3.8). Previous studies substantiate the results obtained with

the TN 90LC family. Deaton et al. (1986) reported no significant differences between populations of 50 lines of inbred KY 16 and 35 ADDH lines of the same variety. Previously, the same author studied ADDH and inbreds of seven burley varieties and concluded that in five of the cultivars, the number of leaves of ADDH lines were not different than the parental cultivars (Deaton et al., 1982). In flue-cured tobacco, no significant differences between ADDH lines and inbred sources of several varieties were also reported in several studies (Arcia et al., 1978; Schnell et al., 1980; Smalcelj et al., 2000). In contrast, other research studies in burley and flue-cured tobaccos support the hypothesis that there are differences between methods used to generate DH lines. Arcia et al. (1978), Kasperbauer et al. (1983), Nielsen and Collins (1989), Wernsman et al. (1989) and Smalcelj & Perica (2000) all reported statistically significant differences for the number of leaves per plant between DH and inbred sources.

The average yield for the inbred check TN 90LC population was 2889 kg/ha, which was 56 kg/ha less (1.94%) than the MDDH TN 90LC population and 112 kg/ha more (3.88%) than the ADDH TN 90LC population (table 3.9). The yield difference between the ADDH and the inbred TN 90LC populations was statistically significant, but the yield difference between TN 90LC and MDDH TN 90LC populations was not significant (table 3.8). There was considerable variation in yield between locations (table 3.7). In comparison to TN 90LC, the TN 90LC ADDH population was lower yielding at the GR, WC, and HR locations, but superior at the LX location. The difference was substantially greater at the GR location (262 Kg/ha).

Poor yielding ability of ADDH lines has been reported in past studies of burley tobacco. 35 ADDH lines of KY 16 yielded 7.3% less Kg/ha of cured leaves and the difference was statistically significant when compared to 50 selfed lines (Deaton et al., 1986). Kasperbauer et al. (1983) also detected lower yielding ability of the ADDH lines compared to the inbred parental sources, in an evaluation of five burley tobacco cultivars. Schnell et al. (1980) also reported the inferiority of ADDH lines, by comparing the yield of ten ADDH lines with lines inbred through SSD method, both descending from one F_1 individual of a cross between (Hicks x Coker 139). The ADDH lines yielded 10.6% less than the DH population, and the ADDH had greater genetic

variability; with some lines having good yielding potential. In a test with the flue-cured hybrid “DH 10”, 47 ADDH lines were compared to the parental hybrid. Three out of those ADDH lines were superior to the hybrid DH 10, but the overall performance was unsatisfactory. There was also highly significant differences between lines of the ADDH population, including differences between DH lines derived from the same haploid source (Smalcelj and Perica, 2000).

TN 90LC family – Comparison of individual lines within methods for obtaining DH lines

To fully evaluate the relative effectiveness of the ADH versus MDH methods for producing DH breeding populations, in addition to evaluating the average performance of ADDH and MDDH TN 90LC lines in comparison to the inbred check, it is important to also look at the performance of individual lines within each method. The ANOVA for the differences between lines within the TN 90 LC, ADDH, and MDDH populations are presented in Table 3.10.
Within both the ADDH and MDDH populations, there were significant differences among individual lines for plant height at the 50th day after transplanting; as expected, there were no differences among the ten iterations of the inbred TN 90LC check. The mean values for plant height, ranked from tallest to shortest, for the 30 individual lines included among the TN 90 LC, ADDH, and MDDH populations are presented in Table 3.11. The ranks for the ten ADDH lines ranged from 8-29; only one of the lines ranked in the top ten, while six of the lines ranked 20th or higher. The ranks for plant height for the ten MDDH lines ranged from 1-30, with six lines ranking in the top ten and three lines having a rank of 20 or higher.

Table 3.10 – Analysis of variance for differences between lines (source of variation) within populations, for six agronomic traits.

Population	Statistic	Height 50	Height topping	Leaf length	Leaf width	Leaf number	Yield
ADDH TN 90LC	MS	616.87	494.9	54.26	32.59	1.75	1041164.4
	F value	2.27*	3.04**	6.79***	11.76***	1.05	12.24***
Inbred TN 90LC	MS	267.91	147.63	12.82	6.03	0.877	387927.1
	F value	0.6	0.84	1.23	1.55	0.77	3.83**
MDDH TN 90LC	MS	4107.3	1230.9	40.58	15.58	6.72	2064789.3
	F value	11.96***	3.91**	3.47**	3.12**	3.82**	16.42***

(*), (**), (***) - Differences significant at P<0.05, 0.01, 0.0001, respectively.

Degrees of Freedom for all three populations of TN 90LC = 9

Table 3.11 – Means separation and ordinal ranking of lines by descending order of plant height at the 50th day after transplant and height after topping of the TN 90LC family.

Line	Method	Height 50 (cm)	Rank *	Height topping (cm)	Rank*
1	ADH	113.9 ab	18th	136.6 abc	17th
2	ADH	100.9 bc	28th	128 c	28th
3	ADH	114.6 ab	17th	141.1 ab	11th
4	ADH	122.1 a	8th	143.4 a	7th
5	ADH	95.5 c	29th	113.9 d	29th
6	ADH	102.8 bc	27th	129.8 bc	27th
7	ADH	119 ab	11th	140.6 abc	12th
8	ADH	107 abc	26th	135.2 abc	22nd
9	ADH	108.4 abc	24th	131.9 abc	25th
10	ADH	110.5 abc	23rd	135 abc	24th
1	Inbred	118.9	12th	137.2	15th
2	Inbred	107.4	25th	131.2	26th
3	Inbred	122.9	7th	143.5	6th
4	Inbred	121.9	9th	138.8	13th
5	Inbred	113.1	19th	135	23rd
6	Inbred	117.3	14th	138.1	14th
7	Inbred	125.3	6th	147	2nd
8	Inbred	117.1	16th	137.1	16th
9	Inbred	111.5	22nd	136.1	18th
10	Inbred	117.3	15th	141.5	9th
1	MDH	121.8 ab	10th	142.3 a	8th
2	MDH	117.8 ab	13th	135.7 a	20th
3	MDH	127.6 ab	2nd	144 a	4th
4	MDH	129.6 a	1st	144 a	5th
5	MDH	57.8 c	30th	102 b	30th
6	MDH	127.1 ab	3rd	141.3 a	10th
7	MDH	125.8 ab	5th	146.3 a	3rd
8	MDH	126.6 ab	4th	147.6 a	1st
9	MDH	111.6 b	20th	135.9 a	19th
10	MDH	111.5 b	21st	135.7 a	21st
	range	71.7		45.6	

(*) Rank includes all three populations, not discriminated by method.

Means within columns of the same method followed by the same letter are not different at the 5% level of significance, based on Fischer's LSD.

Height 50 - TN 90LC ADH: Critical value of t = 2 and LSD = 17.51; TN 90LC MDH: Critical value of t = 2 and LSD = 15.588;

Height topping - TN 90LC ADH: Critical value of t = 2.014 and LSD = 12.85. TN 90LC MDH: Critical value of t = 2.014 and LSD = 17.87.

There were also significant differences for plant height after topping among individual lines within both the ADDH and MDDH populations, but no differences within the TN 90LC check population (table 3.10). The ranks for the ten ADDH lines ranged from 7-28, with only one of the lines ranked in the top ten and six of the lines ranked 20th or higher (table 3.11). The ranks for plant height after topping for the ten MDDH lines ranged from 1-30, with five lines ranking in the top ten and three lines having a rank of 20 or higher.

There were significant differences for leaf length among the ten individual lines within both the ADDH and MDDH populations, but not within the TN 90 LC check population (table 3.10). The ranks for leaf length among the ten ADDH lines ranged from 2-28, with only one of the lines ranked in the top ten and five of the lines ranked 20th or higher. The ranks for leaf length for the ten MDDH lines ranged from 1-29, with four lines ranked in the top ten and five lines ranked 20th or higher (table 3.12). There were also significant differences for leaf width among the ten individual lines within both the ADDH and MDDH populations, but not within the TN 90 LC check population (table 3.10). The ranks for the ten ADDH lines ranged from 5-30, with two of the lines ranked in the top ten and seven of the lines ranked 20th or higher (table 3.12). The ranks for leaf width after topping for the ten MDDH lines ranged from 3-29, with two lines ranking in the top ten and four lines having a rank of twenty or higher.

For individual lines within the three TN 90 LC populations, significant differences for leaf number were noted only in the MDDH population (table 3.10). The ranks for leaf number for the ten MDDH lines ranged from 1-30, with five lines ranked in the top ten and three lines ranked 20th or higher (table 3.13). Although no significant differences for leaf number were detected among individual lines within the ADDH population, the ranks for leaf length ranged from 2-29, with only two of the lines ranked in the top ten and five of the lines ranked 20th or higher.

Table 3.12 – Means separation and ordinal ranking by descending order of leaf length and leaf width of populations of the TN 90LC family.

Line	method	Leaf length (cm)	Rank*	Leaf width (cm))	Rank*
1	ADH	59.4 bc	20th	25.6 cd	24th
2	ADH	56.8 c	28th	24.3 d	28th
3	ADH	59.7 ab	17th	27.5 ab	9th
4	ADH	59.2 bc	22nd	25.4 d	26th
5	ADH	52.3 d	30th	20.5 e	30th
6	ADH	60.8 ab	11th	24.6 d	27th
7	ADH	62.3 a	2nd	27.9 a	5th
8	ADH	60.3 ab	13th	27.1 abc	12th
9	ADH	59.4 abc	19th	25.9 bcd	22nd
10	ADH	59.1 bc	23rd	25.5 cd	25th
1	Inbred	60.4	12th	27	13th
2	Inbred	58.9	25th	26.6	17th
3	Inbred	62.2	3rd	27.6	7th
4	Inbred	59.5	18th	26.2	18th
5	Inbred	62.5	1st	29	1st
6	Inbred	59.9	15th	26.8	16th
7	Inbred	61.8	9th	28.6	2nd
8	Inbred	60.2	14th	27.2	11th
9	Inbred	61.9	8th	28.1	4th
10	Inbred	60.9	10th	27.9	6th
1	MDH	58.6 b	27th	25.7 b	23rd
2	MDH	58.8 ab	26th	26.1 b	20th
3	MDH	62 ab	7th	26.1 b	19th
4	MDH	59.2 ab	21st	26.9 ab	14th
5	MDH	54.7 c	29th	23.1 c	29th
6	MDH	59.8 ab	16th	26 b	21st
7	MDH	62 ab	5th	28.5 a	3rd
8	MDH	58.9 ab	24th	27.6 ab	8th
9	MDH	62.1 a	4th	26.8 ab	15th
10	MDH	62 ab	6th	27.3 ab	10th
	range	10.2		8.5	

(*) Rank includes all three populations, not discriminated by method.

Means within columns of the same method followed by the same letter are not different at the 5% level of significance, based on Fischer's LSD.

Leaf length - ADH: Critical value of t = 2.014 and LSD = 2.8463.

Leaf length - MDH: Critical value of t = 2.014 and LSD = 3.4427.

Leaf width - ADH: Critical value of t = 2.014 and LSD = 1.6769.

Leaf width - MDH: Critical value of t = 2.014 and LSD = 2.2491.

Table 3.13 - Means separation and ordinal ranking by descending order of number of leaves per plant and yield for populations of the TN 90LC family.

Line	Method	Leaves per plant	Rank*	Yield (Kg/ha)	Rank*
1	ADH	20.1	11th	2840 ab	18th
2	ADH	20.4	2nd	2795 ab	19th
3	ADH	20.3	4th	2748 ab	24th
4	ADH	19.5	24th	2842 ab	16th
5	ADH	18.7	29th	1900 c	29th
6	ADH	20	12th	2683 ab	25th
7	ADH	19.4	25th	2883 ab	14th
8	ADH	19.7	19th	2892 a	13th
9	ADH	19.6	22nd	2673 ab	26th
10	ADH	19.6	20th	2644 b	27th
1	Inbred	19.9	13th	2778 cde	21st
2	Inbred	19.6	21st	2756 de	23rd
3	Inbred	20.2	9th	3013 abcd	7th
4	Inbred	19.2	27th	2629 e	28th
5	Inbred	20.2	6th	3271 a	1st
6	Inbred	19.7	16th	2951 bcd	10th
7	Inbred	20.1	10th	3047 ab	3rd
8	Inbred	19.3	26th	2929 bcd	12th
9	Inbred	19.7	17th	3016 abc	6th
10	Inbred	19.9	14th	2883 bcde	15th
1	MDH	20.4 a	3rd	2765 b	22nd
2	MDH	20.6 a	1st	2841 b	17th
3	MDH	20.2 ab	5th	3005 ab	8th
4	MDH	19.9 ab	15th	3024 ab	5th
5	MDH	17.3 c	30th	1687 c	30th
6	MDH	19.5 ab	23rd	2794 b	20th
7	MDH	20.2 ab	8th	2964 ab	9th
8	MDH	19.7 ab	18th	2940 ab	11th
9	MDH	20.2 ab	7th	3025 ab	4th
10	MDH	19 b	28th	3148 a	2nd
	range	3.3		1584	

(*) Rank includes all three populations, not discriminated by method.

Means within columns of the same method followed by the same letter are not different at the 5% level of significance, based on Fischer's LSD.

Leaves per plant - MDH: Critical value of t = 2.014 and LSD = 1.3361.

Yield - ADH: Critical value of t = 1.995 and LSD = 240.72.

Yield - Inbred: Critical value of t = 1.9935 and LSD = 259.16 and

Yield - MDH: Critical value of t = 1.9935 and LSD = 288.59

Significant differences for yield were found among the ten individual lines within each of the three TN 90LC families (table 3.10). It is interesting to note that this was the only variable for which a significant difference was found for the ten iterations within the inbred TN 90 LC check population. The ten TN 90LC check iterations ranged in rank from 1-28, with four ranking in the top 10 and three ranking 20th or higher (table 3.13). The ranks for the ten ADDH lines ranged from 13-29, with five ranked 20th or higher. The ranks for yield among the ten MDDH lines ranged from 2-30, with five lines ranking in the top ten and three lines having a rank of 20 or higher.

TN 90LC family – Direct comparison of each ADDH and MDDH line with the inbred source

One of the objectives of the current research project was to determine if any of the individual ADDH or MDDH lines performed as well or better than the inbred source, even if the mean performance for the ADDH and/or MDDH lines was inferior. Because each of the ADDH and MDDH lines was randomly paired with the inbred source to form an independent triplet, which was randomized within the overall split plot experimental design, independent statistical analyses could be run as a randomized complete block design with three replications at each location.

The statistics for the independent analysis for the TN 90LC families are listed in Tables 3.14 - 3.16, with the trait means for the individual lines presented in Table 3.17. Although location and method effects were statistically significant within each triplet family for many of the variables measured, location X method interactions were statistically significant only for leaf length in triplet ten, and for leaf width and yield for triplet five (table 3.15). Both the ADDH and the MDDH lines in triplet five were visibly off-type and found to be outliers; therefore triplet five was not included in the ANOVA comparing methods for obtaining DH lines. The fact that both the ADDH and the MDDH lines were outliers was by coincidence since individual ADDH and MDDH were randomly paired with the inbred source.

Table 3.14 - Analysis of variance of differences between methods within the lines of the TN 90LC genotype, for the agronomic traits plant height at the 50th day after transplant and plant height after topping.

TN 90LC Triplet	statistics	Height 50 -------- Source of variation --------				Height topping --------- Source of variation ---------			
		loc	rep(loc)	method	Loc x method	loc	rep(loc)	method	loc x method
Line 1	DF	2	6	2	4	2	5	2	4
	MS	6884.07	1467.68	144.81	18.57	1028.00	521.31	99.43	53.27
	F Value	197.3***	42.06***	4.15*	0.53	33.56***	17.02***	3.25	1.74
Line 2	DF	2	6	2	4	2	5	2	4
	MS	2948.25	787.29	656.11	123.18	129.31	273.97	99.95	33.91
	F Value	28.04***	7.49**	6.24*	1.17	1.45	3.06	1.12	0.38
Line 3	DF	2	6	2	4	2	5	2	4
	MS	10087.08	444.55	390.30	81.61	1883.38	160.52	18.29	20.33
	F Value	111.97***	4.93**	4.33*	0.91	19.73**	1.68	0.19	0.21
Line 4	DF	2	6	2	4	2	5	2	4
	MS	7177.46	905.76	171.57	15.34	1100.07	302.40	57.91	33.50
	F Value	115.12***	14.53***	2.75	0.25	20.8**	5.72**	1.1	0.63
Line 5	DF	2	6	2	4	2	5	2	4
	MS	1617.11	1066.42	7170.84	211.49	902.27	1120.93	1931.72	160.36
	F Value	8.25**	5.44**	36.6***	1.08	1.75	2.17	3.74	0.31
Line 6	DF	2	6	2	4	2	5	2	4
	MS	5265.84	181.36	1344.97	80.00	725.05	124.25	277.38	5.32
	F Value	50.93***	1.75	13.01**	0.77	20.01**	3.43*	7.66**	0.15
Line 7	DF	2	6	2	4	2	5	2	4
	MS	10484.74	254.11	128.98	145.26	1827.13	51.27	90.50	39.53
	F Value	132.17***	3.2*	1.63	1.83	34.76***	0.98	1.72	0.75
Line 8	DF	2	6	2	4	2	5	2	4
	MS	6336.84	1088.50	866.05	156.24	721.79	373.96	341.92	73.29
	F Value	80.64***	13.85***	11.02**	1.99	8.06**	4.18*	3.82	0.82
Line 9	DF	2	6	2	4	2	5	2	4
	MS	4229.21	909.39	29.76	89.43	625.97	482.34	58.61	54.56
	F Value	53.23***	11.45**	0.37	1.13	12.76**	9.83**	1.19	1.11
Line 10	DF	2	6	2	4	2	5	2	4
	MS	5044.90	1314.07	120.01	37.68	661.68	706.86	92.90	34.19
	F Value	64.96***	16.92***	1.55	0.49	7.62**	8.15**	1.07	0.39

(*), (**), (***) - Differences between lines significant at P<0.05, 0.01, 0.0001, respectively.

Table 3.15 - Analysis of variance of differences between methods within the lines of the TN 90LC genotype, for the agronomic traits leaf length and leaf width.

TN 90LC Triplet	statistics	Leaf Length				Leaf Width			
		-------- Source of variation --------				--------- Source of variation ---------			
		loc	rep(loc)	method	loc x method	loc	rep(loc)	method	loc x method
Line 1	DF	2	5	2	4	2	5	2	4
	MS	283.02	37.99	6.37	5.07	169.60	19.70	4.44	2.25
	F Value	25.74**	3.46*	0.58	0.46	76.64***	8.9**	2.01	1.02
Line 2	DF	2	5	2	4	2	5	2	4
	MS	374.81	2.53	9.96	11.83	201.28	1.75	11.34	6.65
	F Value	69.63***	0.47	1.85	2.2	100.99***	0.88	5.69*	3.34
Line 3	DF	2	5	2	4	2	5	2	4
	MS	147.40	19.78	11.83	4.31	91.61	8.32	5.91	0.77
	F Value	44.17***	5.93**	3.55	1.29	107.37***	9.76**	6.92*	0.9
Line 4	DF	2	5	2	4	2	5	2	4
	MS	219.32	35.51	0.25	4.75	104.87	7.86	3.99	2.69
	F Value	35.51***	5.75**	0.04	0.77	30.62***	2.3	1.17	0.78
Line 5	DF	2	5	2	4	2	5	2	4
	MS	73.04	17.68	201.01	5.97	76.67	13.54	133.42	4.62
	F Value	18.64**	4.51*	51.3***	1.52	94.98***	16.77***	165.28***	5.72*
Line 6	DF	2	5	2	4	2	5	2	4
	MS	182.64	9.61	4.04	5.06	133.46	3.53	9.66	1.78
	F Value	25.12***	1.32	0.56	0.70	45.67***	1.21	3.31*	0.61
Line 7	DF	2	5	2	4	2	5	2	4
	MS	243.75	6.80	1.01	1.95	155.12	1.06	1.18	0.67
	F Value	22.61**	0.63	0.09	0.18	54.75***	0.37	0.42	0.24
Line 8	DF	2	5	2	4	2	5	2	4
	MS	192.73	9.20	6.51	3.32	112.51	5.15	0.14	1.56
	F Value	40.98***	1.96	1.38	0.71	62.68***	2.87	0.08	0.87
Line 9	DF	2	5	2	4	2	5	2	4
	MS	230.84	12.74	13.39	5.77	131.01	5.79	8.24	2.50
	F Value	18.35**	1.01	1.06	0.46	34.55***	1.53	2.17	0.66
Line 10	DF	2	5	2	4	2	5	2	4
	MS	281.82	3.84	16.52	9.06	164.52	4.84	12.17	3.07
	F Value	112.57***	1.53	6.6*	3.62*	140.34***	4.12*	10.38**	2.62

(*), (**), (***) - Differences between lines significant at P<0.05, 0.01, 0.0001, respectively.

Table 3.16 - Analysis of variance of differences between methods within the lines of the TN 90LC genotype, for the traits number of leaves per plant and yield.

TN 90LC Triplet	statistics	Number of leaves per plant -------- Source of variation --------				Yield --------- Source of variation ---------			
		loc	rep(loc)	method	loc X method	loc	rep(loc)	method	loc X method
	DF	2	5	2	4	2	5	2	4
Line 1	MS	0.15	1.58	0.73	0.55	455624.3	240675.7	15719.2	28903.4
	F Value	0.20	2.07	0.96	0.72	4.86*	2.57	0.17	0.31
	DF	2	5	2	4	2	5	2	4
Line 2	MS	1.32	1.48	2.29	0.68	590256.8	161825.9	27179.2	128241.3
	F Value	1.07	1.2	1.86	0.55	8.67**	2.38	0.4	1.88
	DF	2	5	2	4	2	5	2	4
Line 3	MS	1.13	0.84	0.03	0.42	517605.71	170883.76	274143.2	106347.50
	F Value	0.89	0.67	0.03	0.33	7.55**	2.49	4*	1.55
	DF	2	5	2	4	2	5	2	4
Line 4	MS	9.81	1.27	0.70	1.20	597385.94	186820.4	469704.9	186766.87
	F Value	13.21**	1.71	0.94	1.62	7.21**	2.26	5.67*	2.26
	DF	2	5	2	4	2	5	2	4
Line 5	MS	7.08	3.95	14.78	1.13	369370.57	75582.81	8867065.	307849.72
	F Value	1.88	1.05	3.93*	0.30	6.12**	1.25	146.97***	5.1**
	DF	2	5	2	4	2	5	2	4
Line 6	MS	2.22	0.56	0.59	0.98	991536.99	64106.19	217389.5	131875.43
	F Value	2.43	0.61	0.64	1.07	10.14**	0.66	2.22	1.35
	DF	2	5	2	4	2	5	2	4
Line 7	MS	4.11	3.11	1.33	0.70	860410.81	124882.44	80502.02	102653.20
	F Value	4.46*	3.38*	1.44	0.77	10**	1.45	0.94	1.19
	DF	2	5	2	4	2	5	2	4
Line 8	MS	6.79	4.94	0.55	0.35	432626.21	100399.69	7727.30	92790.91
	F Value	7.5*	5.45*	0.61	0.39	4.42*	1.02	0.08	0.95
	DF	2	5	2	4	2	5	2	4
Line 9	MS	4.08	2.05	0.75	0.18	470582.68	97222.27	663961.3	199121.00
	F Value	4.9*	2.46	0.90	0.21	2.50	0.52	3.52	1.06
	DF	2	5	2	4	2	5	2	4
Line 10	MS	3.11	3.71	1.30	0.29	866562.09	171499.63	761631.1	147521.98
	F Value	1.92	2.29	0.80	0.18	6.71**	1.33	5.9*	1.14

(*), (**), (***) - Differences between lines significant at P<0.05, 0.01, 0.0001, respectively.

Table 3.17 – Agronomic trait means for lines of the TN 90LC family and separation of means within triplets based on Fischer's LSD.

TN 90LC triplet	Method	Height 50 (cm)		Height topping (cm)		Leaf length (cm)		Leaf width (cm)		Leaf number (No)		Yield (Kg/ha)	
Line 1	ADH	113.9	b	136.6	a	59.4	a	25.6	a	20	a	2840	a
	Inbred	118.9	ab	137.2	a	60.4	a	27	a	19.9	a	2778	a
	MDH	121.8	a	142.3	a	58.6	a	25.7	a	20.4	a	2765	a
Line 2	ADH	100.9	b	128	a	56.8	a	24.3	b	20.4	a	2795	a
	Inbred	107.4	ab	131.2	a	58.9	a	26.6	a	19.6	a	2756	a
	MDH	117.8	a	135.7	a	58.8	a	26.1	a	20.6	a	2841	a
Line 3	ADH	114.6	b	141.1	a	59.7	b	27.5	a	20.3	a	2748	b
	Inbred	122.9	ab	143.5	a	62.2	a	27.6	a	20.2	a	3013	a
	MDH	127.6	a	144	a	62	a	26.1	b	20.2	a	3005	a
Line 4	ADH	122.1	a	143.4	a	59.2	a	25.4	a	19.5	a	2842	ab
	Inbred	121.9	a	138.8	a	59.5	a	26.2	a	19.2	a	2629	b
	MDH	129.6	a	144	a	59.2	a	26.9	a	19.9	a	3024	a
Line 5	ADH	95.5	b	113.9	ab	52.3	c	20.5	c	18.7	ab	1900	b
	Inbred	113.1	a	135	a	62.5	a	29	a	20.2	a	3271	a
	MDH	57.8	c	102	b	54.7	b	23.1	b	17.3	b	1687	c
Line 6	ADH	102.8	b	129.8	b	60.7	a	24.6	b	20	a	2683	a
	Inbred	117.3	a	138.1	a	59.9	a	26.8	a	19.7	a	2951	a
	MDH	127.1	a	141.3	a	59.8	a	26	ab	19.5	a	2794	a
Line 7	ADH	119	a	140.6	a	62.3	a	27.9	a	19.4	a	2883	a
	Inbred	125.3	a	147	a	61.8	a	28.6	a	20.1	a	3047	a
	MDH	125.8	a	146.3	a	62	a	28.5	a	20.2	a	2964	a
Line 8	ADH	107	c	135.2	b	60.3	a	27.1	a	19.7	a	2892	a
	Inbred	117.1	b	137.1	b	60.2	a	27.2	a	19.3	a	2929	a
	MDH	126.6	a	147.6	a	58.9	a	27.6	a	19.7	a	2940	a
Line 9	ADH	108.4	a	131.9	a	59.4	a	25.9	b	19.6	a	2673	a
	Inbred	111.5	a	136.1	a	61.9	a	28.1	a	19.7	a	3016	a
	MDH	111.6	a	135.9	a	62.1	a	26.8	ab	20.2	a	3025	a
Line 10	ADH	110.5	a	135	a	59.0	b	25.5	b	19.6	a	2644	b
	Inbred	117.3	a	141.5	a	61.0	a	27.9	a	19.9	a	2883	ab
	MDH	111.5	a	135.7	a	62.0	a	27.3	a	19	a	3148	a

Means followed by the same letter within a column and within a TN 90LC line are not significantly different at the 5% level of significance.

Because location X method interactions were statistically significant only for leaf length in triplet ten when triplet five was not considered, the data for methods were pooled across locations. Among the ADDH lines; lines one, four, and seven were not significantly different from the TN 90LC source for any agronomic trait measured. Significant differences from the inbred source were detected only for leaf width for line two and for plant height 50 days after transplanting for line eight. Although the differences were not statistically significant, lines one, two, and four produced higher yields than the inbred TN 90LC in adjacent rows. Significant differences between the ADDH line and TN 90LC inbred source were found for leaf length and yield for line three; height 50 and leaf width for line six; leaf width for line nine; and leaf length and width for line ten. Although not statistically significant, lines six, nine, and ten were substantially lower yielding than TN 90LC in adjacent rows. ADDH line five was significantly different from TN 90LC for height 50, leaf length, and leaf width. A highly significant difference was observed for yield, with the yield of ADDH line five being 42% lower than for TN 90LC.

Among the TN 90LC MDDH lines; lines one, two, six, seven, nine, and ten were not significantly different from TN 90LC for any agronomic trait measured. Significant differences from TN 90LC were found for leaf width for line three, and for plant height 50 days after transplanting and at topping for line eight. Among these eight lines, lines two, eight, nine, and ten produced yields higher than TN 90LC in adjacent rows, but these difference were not statistically significant. However, Line 4 did produce a significantly higher yield than the adjacent TN90LC source. Significant differences were found between MDDH line 5 and TN 90LC for every agronomic trait measured.

Nielsen and Collins (1989) reported results similar to the performance of TN 90LC ADDH line 5 and MDDH line 5 in the current study. They found that in a comparison of four MDDH, four ADDH and the inbred sources derived from four different plants of KY 17, inferiority of both DH methods were detected in one MDDH and in one ADDH line, which yielded 18.7% and 18.9% less than the inbred, respectively. The other three lines of each method were not statistically significant different than the source variety. Their research showed an equivalent performance of ADDH and MDDH lines for several agronomic traits, with similar yield ability and comparable levels of

variation. Like what was detected in the TN 90LC experiment, this is an example of the variation existing in a DH population; this variation sometimes is hidden by the mean of the populations, because the variability is evenly distributed around the average.

GR 149LC family – Location effects

As observed for the trials of the TN 90LC family, there were statistically significant differences between the GR, HR and WC locations for all six agronomic traits measured (table 3.18). The differences between locations for all agronomic traits are shown in table 3.19. Comparing the performance of all six traits of the three GR 149LC populations, the HR location ranked last in all population x trait combinations (except for the number of leaves per plant in the ADDH and inbred check populations). The WC location ranked first in all three populations for plant height after topping and number of leaves per plant. The GR location was superior for the traits leaf length, leaf width and yield. The GR location was superior to the WC location, which in turn, was superior to the HR location for all three methods. For all three methods, the GR locations produced yields that were more than 1,000 Kg/ha higher than the HR location (table 3.19).

GR 149LC Family – Comparison of methods for obtaining DH lines

The ANOVA for differences among methods of generating haploid lines of GR 149LC is presented in Table 3.20. On average over the three locations, the plant height at the 50th day after transplanting was significantly different among all of the three methods. The plant height at the 50th day after transplant of the inbred population was 76.64 cm, 4 cm (5.2%) taller than the ADDH GR 149LC population, and 3.37 cm (4.4%) taller than the MDDH GR 149LC population (table 3.21). The average height of the four plants comprising the MDDH and the eight plants of the ADDH GR 149LC populations were inferior to the inbred GR 149LC check population.

Table 3.18 - Analysis of variance of differences among locations for the GR 149LC trial.

Statistic	Height 50	Height topping	Leaf length	Leaf width	Leaf Number	Yield
DF	2	2	2	2	2	2
MS	556.64	3069.57	6149.68	1627.09	104.61	20394351.33
F value	16.90***	73.98***	1112.1***	569.36***	91.43***	297.73***

(*), (**), (***) - Differences between lines significant at P<0.05, 0.01, 0.0001, respectively.

Table 3.19 – Agronomic traits means for the GR 149LC family by location.

Trait	Location	---------- methods ----------		
		ADDH	Inbred	MDDH
Height 50 (cm)	GR	73.4	77.7	72.4
	HR	68.3	74.8	70.6
	WC	76.1	77.4	76.8
Height topping (cm)	GR	126.9	129.4	131.1
	HR	123.2	126.6	124.2
	WC	136.4	137.7	139.5
Leaf length (cm)	GR	67.3	68.0	67.3
	HR	49.0	48.9	49.3
	WC	60.9	60.9	59.3
Leaf width (cm)	GR	32.7	31.0	32.7
	HR	22.9	22.0	23.1
	WC	29.7	28.2	29.6
Number of leaves per plant (No)	GR	18.9	20.5	19.5
	HR	19.7	20.8	19.4
	WC	21.8	22.2	21.8
Yield (Kg/ha)	GR	3883	3842	3806
	HR	2711	2810	2793
	WC	3478	3401	3430

This was the same trend observed for the ADDH TN 90LC population, but the opposite of what was observed for TN 90LC MDDH population. The MDDH GR 149LC population, and the ADDH and MDDH populations of the TN 90LC, displayed significant differences between lines for plant height at the 50th day after transplant. The eight plants of the ADDH GR 149LC populations were considerably homogeneous for this trait.

The results of the present study are in accord with the findings in the flue-cured tobacco varieties NC 95 and Coker 139 by Arcia et al. (1978). The same trend was not observed in oriental tobacco varieties; in a comparison between ADDH lines and inbred sources of three oriental tobacco varieties, Miceska (2009) reported superiority of ADDH method over the inbred populations in two of the varieties tested, while one inbred population of oriental tobacco was taller than the ADDH lines derived from it.

For plant height after topping, there were no statistically significant differences detected among MDDH GR 149LC and the inbred check (table 3.20); these populations were just 0.3% different (table 3.21). Statistically significant differences were detected between ADDH GR 149LC and the inbred population, with the ADDH population being 1.8% shorter (table 3.21). For the plant height after topping, the MDDH populations of the TN 90LC and GR 149LC were taller than the inbred checks, but only the difference between MDDH TN 90LC and the respective inbred populations was significant. Nielsen and Collins, (1989) observed that three out of the four five-lines sets of KY 17 MDDH were also not significant different from inbred source lines and one set was actually inferior to the inbred population. As observed in the GR 149LC and TN 90LC experiments, the MDDH populations were significantly taller for plant height after topping, compared to the inbred sources. The ADDH populations of both genotypes were shorter, but just the TN 90LC ADDH population was significant different.

For leaf length, there were no statistically significant differences between any of the populations (table 3.20). The average leaf length of ADDH GR 149LC, inbred check GR 149LC and MDDH GR 149LC populations was 59.1, 58.6cm and 59.3 cm, respectively (table 3.21).

Table 3.20 - Analysis of variance of differences among GR 149LC methods (source of variation).

Comparison	Statistics	Height 50	Height topping	Leaf length	Leaf width	Leaf Number	Yield
Between All three populations	DF	2	2	2	2	2	2
	MS	318.74	217.76	0.67	32.34	20.07	1919.16
	F value	9.68**	5.25**	0.12	11.32***	17.54***	0.03
Inbred x ADDH	DF	1	1	1	1	1	1
	MS	581.68	204.64	1.33	64.67	37.72	1529.81
	F value	14.89**	5.41*	0.32	28.34***	36.7***	0.02
Inbred x MDDH	DF	1	1	1	1	1	1
	MS	468.69	2.01	8.56	62.83	30.37	7075.34
	F value	17.05**	0.05	1.38	22.7***	24.35***	0.1

(*), (**), (***) - Differences between lines significant at P<0.05, 0.01, 0.0001, respectively.

Table 3.21 - Mean, standard deviation, standard error, coefficient of variation, minimum and maximum values for inbred GR 149LC and ADDH and MDDH populations over three locations - 2014 (GR, HR and WC).

Statistics	Height 50 (cm)	Height topping (cm)	Leaf length (cm)	Leaf width (cm)	Leaves per plant	Yield (Kg/ha)
---------- ADDH GR 149LC population ----------						
Mean	72.62	128.84	59.07	28.43	20.15	3357
Std Dev	9.29	11.14	8.63	5.64	2.62	569.69
Std Error	1.1	1.31	1.02	0.67	0.31	67.14
CV	0.13	0.09	0.15	0.2	0.13	0.17
Min	52.83	106.17	42.17	16.83	15.83	2225
Max	96	156.33	74.67	39.83	27.33	4394
---------- Inbred GR 149LC population ----------						
Mean	76.64	131.22	59.26	27.09	21.17	3351
Std Dev	8.03	9	8.64	4.28	2.13	508.23
Std Error	0.95	1.06	1.02	0.5	0.25	59.89
CV	0.1	0.07	0.15	0.16	0.1	0.15
Min	58.13	111.33	42.67	19.17	15.83	2372
Max	98.63	151.5	72.5	34	26	4334
---------- MDDH GR 149LC population ----------						
Mean	73.27	131.62	58.63	28.43	20.24	3343
Std Dev	7.73	10.36	8.45	5.64	2.24	527.49
Std Error	0.91	1.22	1	0.66	0.26	62.16
CV	0.11	0.08	0.14	0.2	0.11	0.16
Min	54.38	99.83	34.17	18	16.5	2349
Max	90.88	151.67	72	38.67	26.67	4402

The ANOVA revealed statistically significant differences for leaf width between the ADDH GR 149LC and the inbred check GR 149LC population (table 3.20). The average leaf width of the inbred GR 149LC check population was 27.1 cm and the width of leaves of both ADDH and MDDH GR 149LC populations was 28.4 cm (table 3.21). The difference in the leaf width was almost 5% among the DH populations and the inbred GR 149LC. However, since there were statistically significant interactions between location, line and method (Table A 3.1 in the appendix), the four lines of MDDH GR 149LC population should not be considered different than the inbred check GR 149LC.

According to the ANOVA, the number of leaves per plant of both DH methods was significantly different from the number of leaves of the inbred GR 149LC population (table 3.20). The ADDH and the MDDH GR 149LC populations had an average of 20.15 and 20.24 leaves per plant, respectively (table 3.21). Those values represent a difference of 4.8% and 4.4% fewer leaves per plant than the inbred check GR 149LC.

No statistically significant differences for yield of cured leaves between the three different methods were detected by the ANOVA (table 3.20). The ADDH GR 149LC was the highest yielding population, producing 3357 Kg/ha, the inbred GR 149LC population yielded 3351 Kg/ha and the MDDH GR 149LC, 3343 Kg/ha (table 3.21).

In a comparison of four MDDH, four ADDH and the inbred sources derived from four different plants of KY 17, inferiority of both DH methods were detected in one MDDH and one ADDH line, which yielded 18.7% and 18.9% less than the inbred, respectively (Nielsen and Collins, 1989). The other three lines of each method were not statistically significant different than the source variety. The research showed an equivalent performance of ADDH and MDDH lines for several agronomic traits, with similar yields ability and comparable levels of variation. Deaton et al. (1982) obtained similar results studying seven burley varieties; they reported no significant differences in yield between ADDH and the inbred parental sources in five of the seven varieties compared.

There are plenty of examples in the literature about the inferiority of the ADDH method compared to inbred sources and MDDH technique. The results

obtained in the TN 90LC experiment corroborate those findings, but the trend was not observed in the GR 149LC trials. Studies using several genotypes and the respective DH lines showed that there are situations where the ADDH population is comparable or even superior to the inbred sources. In a test with the flue-cured hybrid “DH 10”, 47 ADDH lines were compared to the parental hybrid. Three of those ADDH lines were superior to the hybrid DH 10, but the overall performance was unsatisfactory (Smalcelj and Perica, 2000). In a study with three different oriental tobacco cultivars in Southeast Europe, two of the diploid sources were superior to the average yield of their ADDH lines. In one of the genotypes, the ADDH lines were superior to the source cultivar, in 14.2 % (Miceska, 2009).

GR 149LC family - Comparison of individual lines within methods for obtaining DH lines

The ANOVA comparing height of the lines within the ADDH and lines within the inbred check GR 149LC populations did not detect any significant differences for plant height at the 50th day after transplant, but statistically significant differences were revealed in the MDDH GR 149LC population (table 3.22). For plant height after topping, no significant differences were observed between lines within the three populations (table 3.22).

The mean values for plant height at the 50th day after transplant and plant height after topping, ranked from tallest to shortest, for the 20 individual lines included among the ADDH, MDDH and the inbred check GR 149LC populations are presented in Table 3.23. The ranks for plant height at the 50th day after transplant among the eight ADDH lines ranged from 5-20, with only two of the lines ranked in the top ten and four of the lines ranked 15th or higher. The ranks for plant height at the 50th day after transplant for the four MDDH lines ranged from 4-18, with just one line ranked in the top ten and two lines ranked 15th or higher. For plant height after topping, the ranks of ADDH lines ranged from 4-20, with four lines among the top ten, but half of the population ranked 17th or higher.

Table 3.22 – Analysis of variance for differences between lines (source of variation) within populations, for six agronomic traits.

Population	Statistic	Height 50	Height topping	Leaf length	Leaf width	Leaf number	Yield
ADDH GR 149LC	MS	62.82	83	47.47	98.43	5.67	112415.7
	F value	1.28	1.16	7.66***	32.91***	3.32**	1.54
Inbred GR 149LC	MS	35.45	19.13	6.58	3.82	1.73	66004.9
	F value	0.73	0.46	1.09	1.66	1.72	0.79
MDDH GR 149LC	MS	163.06	73.86	41.06	251.66	6.37	335614.4
	F value	6.14**	1.40	5.04**	76.44***	4.21**	4.98**

(*), (**), (***) - Differences between lines significant at P<0.05, 0.01, 0.0001, respectively.

Degrees of Freedom for ADDH GR 149LC = 7;

Degrees of Freedom for MDDH GR 149LC = 3;

The MDH method had a limited number of lines, and they ranged from 1-16. Two of the lines ranked in the top ten and one was ranked 15th or higher (table 3.23).

The length of leaves of lines within both ADDH and MDDH GR 149LC populations were significantly different from others of the same population (table 3.22). Since these two methods showed significant differences within populations, means separation for leaf length of the two populations are presented in Table 3.24. The leaf length ranks for the eight ADDH lines ranged from 1-20, with four of the lines ranked in the top ten and four of the lines ranked 15th or higher. For the leaf width trait, lines within the respective populations were statistically different than others within the respective ADDH and MDDH GR 149LC populations (table 3.22). The ranks for leaf width for the four MDDH lines ranged from 3-19, with half of the lines ranking in the top ten and the other half of the lines having a rank of eighteen or higher (table 3.24). The leaf width ranks for the eight ADDH lines ranged from 2-20, with five of the lines ranked in the top ten; conversely one line was the worst performing among all twenty lines across the three methods. The ranks for the four MDDH lines ranged from 1-19, with three of the lines ranking in the top ten. One of the lines ranked 19th among all twenty lines across the three methods.

For number of leaves per plant, both the ADDH and MDDH populations displayed statistically significant differences among lines within each of those populations (table 3.22). MDDH TN 90LC was the only population in which lines were statistically different from others for yield (table 3.22). Mean values and ranks for both the number of leaves per plant and yield are displayed in Table 3.25. The ranks for the number of leaves per plant for the ADDH lines ranged from 2-20, with just two lines ranked in the top ten and four lines ranked 15th or higher. The ranks for leaf number for the MDDH lines ranged from 9-17, with only one of the lines ranked in the top ten and just one line ranked 15th or higher. For the yield of cured leaves per hectare, the eight ADDH GR 149LC lines ranged from 1-20, with three ranking in the top ten and two ranking 15th or higher. The ranks for yield among the four MDDH lines ranged from 2-19, with two lines ranking in the top ten and two lines having a rank of fifteen or higher (table 3.25).

Table 3.23 – Means separation and ordinal ranking of lines by descending order of the plant height at the 50th day after transplant and plant height after topping of the GR 149LC family.

Line	Method	Height 50 (cm)	Rank*	Height topping (cm)	Rank*
1	ADH	75	10th	130.5	9th
2	ADH	69.9	19th	127.4	18th
3	ADH	70.7	17th	123.4	20th
4	ADH	69.8	20th	126.1	19th
5	ADH	72.8	14th	130.5	10th
6	ADH	71.3	16th	129.4	17th
7	ADH	76.9	5th	132.8	4th
8	ADH	74.7	11th	130.6	8th
1	Inbred	75.3	9th	133.6	2nd
2	Inbred	79	1st	130.3	11th
3	Inbred	78.5	2nd	129.9	13th
4	Inbred	78	3rd	131.5	7th
5	Inbred	76.3	8th	132.2	6th
6	Inbred	76.8	6th	132.6	5th
7	Inbred	76.5	7th	129.9	14th
8	Inbred	72.8	13th	129.9	15th
1	MDH	70.4 b	18th	129.7	16th
2	MDH	77.5 a	4th	133.7	1st
3	MDH	72.9 b	12th	133	3rd
4	MDH	72.3 b	15th	130.1	12th
	range	9.2		10.3	

(*) Rank includes all three populations, not discriminated by method.

Means within columns of the same method followed by the same letter are not different at the 5% level of significance, based on Fischer's LSD.

Height 50 - GR 149LC MDH: Critical value of t = 2 and LSD = 3.4431.

Table 3.24 – Means separation and ordinal ranking of lines by descending order of leaf length and leaf width of the populations of the GR 149LC family.

Line	method	Leaf length (cm)	Rank*	Leaf width (cm)	Rank*
1	ADH	59.9 b	6th	27.1 b	13th
2	ADH	57.8 bc	17th	31.7 a	2nd
3	ADH	58.2 bc	15th	31.4 a	3rd
4	ADH	56.1 c	20th	30.1 a	6th
5	ADH	58 bc	16th	30.6 a	5th
6	ADH	60 b	5th	27.8 b	9th
7	ADH	63.9 a	1st	21.7 c	20th
8	ADH	58.8 b	10th	27.1 b	14th
1	Inbred	58.7	12th	26.4	17th
2	Inbred	59.7	7th	27.4	10th
3	Inbred	60.1	4th	27.1	12th
4	Inbred	58.5	14th	26.4	18th
5	Inbred	58.6	13th	27	15th
6	Inbred	59.1	8th	27.3	11th
7	Inbred	58.7	11th	26.8	16th
8	Inbred	60.9	2nd	28.4	7th
1	MDH	57.4 b	19th	31.7 a	1st
2	MDH	58.9 ab	9th	23.4 c	19th
3	MDH	60.6 a	3rd	27.9 b	8th
4	MDH	57.6 b	18th	30.7 a	4th
	range	7.8		10	

(*) Rank includes all three populations, not discriminated by method.

Means within columns of the same method followed by the same letter are not different at the 5% level of significance, based on Fischer's LSD.

Leaf length – ADH method: Critical value of t = 2.018 and LSD = 2.368 and MDH method: Critical value of t = 2.005 and LSD = 1.9072. Leaf width - ADH method: Critical value of t = 2.018 and LSD = 1.6454 and MDH method: Critical value of t = 2.005 and LSD = 1.213

Table 3.25 - Means separation and ordinal ranking by descending order of the number of leaves per plant and yield of the GR 149LC family.

Line	Method	Leaves per plant	Rank*	yield	Rank*
1	ADH	19.7 b	15th	3274	16th
2	ADH	19.5 b	20th	3337	13th
3	ADH	19.7 b	16th	3406	7th
4	ADH	19.6 b	18th	3349	12th
5	ADH	20.5 ab	11th	3470	3rd
6	ADH	19.5 b	19th	3356	11th
7	ADH	21.5 a	2nd	3155	20th
8	ADH	21.1 a	8th	3512	1st
1	Inbred	21.4	5th	3407	6th
2	Inbred	21.2	6th	3394	9th
3	Inbred	21.1	7th	3206	18th
4	Inbred	21.8	1st	3433	5th
5	Inbred	21.4	3rd	3308	14th
6	Inbred	21.4	4th	3441	4th
7	Inbred	20.7	10th	3259	17th
8	Inbred	20.4	12th	3359	10th
1	MDH	20 bc	14th	3492 a	2nd
2	MDH	21 a	9th	3172 c	19th
3	MDH	20.4 ab	13th	3308 bc	15th
4	MDH	19.6 c	17th	3400 ab	8th
	range	2.4		357	

(*) Rank includes all three populations, not discriminated by method.

Means within columns of the same method followed by the same letter are not different at the 5% level of significance, based on Fischer's LSD.

Leaf number - ADH method: Critical value of t = 2.018 and LSD = 1.2439 and MDH method: Critical value of t = 2.005 and LSD = 0.8214.

Yield - MDH method: Critical value of t = 2.005 and LSD = 173.51

GR 149LC family - Direct comparison of each ADDH and MDDH line with the inbred source

The statistics for the direct comparisons of individual ADDH or MDDH lines with their GR 149LC source are presented in Tables 3.26 - 3.28. As observed for the TN 90LC study, although location and method effects were statistically significant within each triplet family for many of the variables measured, location X method interactions were statistically significant for a very limited number of observations. The location X method interactions that were statistically significant included: plant height at the 50th day after transplant for triplets seven and eight and plant height at topping for triplet eight (table 3.26); leaf width for triplets one and two (table 3.27); and yield for triplet two (table 3.28).

Because location X method interactions were statistically significant for only six out of 48 total observations and were typically a result of magnitude rather than rank, the data for methods were pooled across locations. Mean values for the individual lines are presented in Table 3.29. Among the eight ADDH lines, line eight was not significantly different from the GR 149LC source for any agronomic trait measured. Lines one and six were significantly different from the inbred source only for leaf number. Line five had a significantly wider leaf than the source; conversely, the leaf width of line seven was significantly smaller than GR 149LC. Lines two, three and four were significantly shorter than the inbred source for plant height at the 50th day after transplant; however, the shorter plant height persisted at topping only for lines three and four. In comparison to the inbred source, lines two, three and four also had fewer but wider leaves, with leaves of line two also being significantly shorter than for GR 149LC. Most importantly, none of the eight ADDH lines were significantly lower yielding than the inbred source. Although the differences were not statistically significant, lines four, five and eight produced higher yields than did GR 149LC.

Table 3.26 - Analysis of variance of differences between methods within the lines of the GR 149LC population, for the agronomic traits plant height at the 50th day after transplant and plant height after topping.

Triplet	statistics	Height 50				Height topping			
		--------- Source of variation ----------				---------- Source of variation ----------			
		loc	rep(loc)	method	loc x method	Loc	rep(loc)	method	loc x method
	DF	2	6	2	4	2	6	2	4
Line 1	MS	154.20	293.90	98.28	28.33	612.29	329.74	47.10	34.76
	F Value	4.88*	9.3***	3.11	0.9	9.61**	5.17**	0.74	0.55
	DF	2	6	2	4	2	6	2	4
Line 2	MS	129.87	152.60	228.84	58.37	522.71	172.85	124.07	17.94
	F Value	2.69	3.16*	4.74*	1.21	22.78***	7.53**	5.41*	0.78
	DF	2	6	2	4	2	6	2	4
Line 3	MS	26.79	114.82	150.76	51.57	553.07	146.14	281.44	24.76
	F Value	1.12	4.8**	6.3**	2.16	11.24**	2.97*	5.72*	0.5
	DF	2	6	2	4	2	6	2	4
Line 4	MS	49.23	197.44	161.21	14.10	580.23	245.425	72.79	21.74
	F Value	1.62	6.48**	5.29*	0.46	17.86***	7.56**	2.24	0.67
	DF	2	6	1	2	2	6	1	2
Line 5	MS	52.03	149.72	54.25	0.68	280.68	94.96	12.50	0.82
	F Value	1.67	4.81*	1.74	0.02	8.73*	2.95	0.39	0.03
	DF	2	6	1	2	2	6	1	2
Line 6	MS	91.56	63.15	136.18	25.35	185.40	115.05	45.09	47.70
	F Value	1.15	0.8	1.72	0.32	3.42	2.12	0.83	0.88
	DF	2	6	1	2	2	6	1	2
Line 7	MS	99.26	92.50	0.64	252.64	190.37	130.21	38.49	24.36
	F Value	14.4**	13.42**	0.09	36.66**	3.64	2.49	0.74	0.47
	DF	2	6	1	2	2	6	1	2
Line 8	MS	32.96	181.42	15.13	46.55	248.02	264.06	2.74	124.66
	F Value	6.01*	33.09**	2.76	8.49*	13.12**	13.97**	0.14	6.6*

(*), (**), (***) - Differences between lines within the same triplet significant at P<0.05, 0.01, 0.0001, respectively.

Table 3.27 - Analysis of variance of differences between methods within the lines of the GR 149LC population, for the agronomic traits leaf length and leaf width.

Triplets	statistics	Leaf Length				Leaf Width			
		--------- Source of variation ---------				--------- Source of variation ---------			
		loc	rep(loc)	method	loc x method	Loc	rep(loc)	method	loc x method
	DF	6	2	4		2	6	2	4
Line 1	MS	1018.70	37.57	20.00	9.54	316.50	4.88	113.93	7.53
	F Value	211.74***	7.81**	4.16*	1.98	125.68***	1.94	45.24***	2.99*
	DF	2	6	2	4	2	6	2	4
Line 2	MS	834.22	52.44	7.98	8.99	246.85	5.46	215.15	14.05
	F Value	193.07***	12.14***	1.85	2.08	87.68***	1.94	76.42***	4.99**
	DF	2	6	2	4	2	6	2	4
Line 3	MS	1006.38	35.77	18.23	6.19	295.04	10.03	49.03	2.68
	F Value	139.04***	4.94**	2.52	0.86	107.99***	3.67*	17.95***	0.98
	DF	2	6	2	4	2	6	2	4
Line 4	MS	734.55	78.98	13.23	9.46	231.55	14.87	58.28	5.57
	F Value	100.36***	10.79***	1.81	1.29	46.81***	3.01*	11.78**	1.13
	DF	2	6	1	2	2	6	1	2
Line 5	MS	598.36	17.95	2.00	2.79	216.76	9.50	56.96	3.10
	F Value	161.12***	4.83*	0.54	0.75	136.8***	5.99*	35.95**	1.95
	DF	2	6	1	2	2	6	1	2
Line 6	MS	656.29	28.68	3.71	1.46	149.17	8.31	1.30	1.54
	F Value	96.8***	4.23	0.55	0.22	59.85**	3.34	0.52	0.62
	DF	2	6	1	2	2	6	1	2
Line 7	MS	510.81	26.45	120.90	4.71	54.51	4.43	116.64	4.02
	F Value	226.4***	11.72**	53.59**	2.09	59.33***	4.82*	126.94***	4.37
	DF	2	6	1	2	2	6	1	2
Line 8	MS	551.87	25.98	19.32	4.68	121.20	11.65	8.43	3.67
	F Value	142.72***	6.72*	5.00	1.21	130.03***	12.49**	9.05*	3.93

(*), (**), (***) - Differences between lines within the same triplet significant at P<0.05, 0.01, 0.0001, respectively.

Table 3.28 - Analysis of variance of differences between methods within the lines of the GR 149LC population, for the agronomic traits number of leaves per plant and yield.

Triplets	statistics	Leaf Number				Yield			
		---------- Source of variation ----------				--------- Source of variation ----------			
		loc	rep(loc)	method	loc x method	Loc	rep(loc)	method	loc x method
Line 1	DF	2	6	2	4	2	6	2	4
	MS	29.11	25.46	7.41	0.53	3654837.7	145706.9	142543.9	17024.3
	F Value	26.04***	22.77***	6.63**	0.47	56.6***	2.26	2.21	0.26
Line 2	DF	2	6	2	4	2	6	2	4
	MS	11.63	16.90	8.60	1.05	2223193.7	59275.3	176525.8	369702.8
	F Value	11.86**	17.24***	8.77**	1.07	28.02***	0.75	2.23	4.66**
Line 3	DF	2	6	2	4	2	6	2	4
	MS	27.60	20.82	4.14	1.62	3256023.8	34915.7	90421.3	85304.2
	F Value	27.51***	20.76***	4.13*	1.61	47.78***	0.51	1.33	1.25
Line 4	DF	2	6	2	4	2	6	2	4
	MS	11.91	10.68	16.68	0.48	4939162.7	98389.6	16476.8	71635.6
	F Value	8.3**	7.44**	11.62**	0.34	83***	1.65	0.28	1.2
Line 5	DF	2	6	1	2	2	6	1	2
	MS	4.95	15.46	3.70	1.08	2509758.7	72717.4	119140.8	158170.3
	F Value	4.58	14.3**	3.42	1.00	25.46**	0.74	1.21	1.6
Line 6	DF	2	6	1	2	2	6	1	2
	MS	8.02	7.91	15.16	0.30	1701848.5	60216.8	32796.3	77337.8
	F Value	5.29*	5.21*	10*	0.20	30.45**	1.08	0.59	1.38
Line 7	DF	2	6	1	2	2	6	1	2
	MS	8.90	6.62	3.26	3.00	887510.3	92269.0	48903.1	98524.8
	F Value	6.65*	4.95*	2.44	2.24	28.42**	2.95	1.57	3.15
Line 8	DF	2	6	1	2	2	6	1	2
	MS	10.01	12.77	1.79	3.90	1943894.7	150087.7	104447.5	16966.8
	F Value	12.81**	16.34**	2.29	4.99	20.07**	1.55	1.08	0.18

(*), (**), (***) - Differences between lines within the same triplet significant at P<0.05, 0.01, 0.0001, respectively.

Table 3.29 – Agronomic trait means for lines of the GR 149LC family and separation of means within triplets based on Fischer's LSD.

Triplet	Method	Height 50 (cm)		Height topping (cm)		Leaf length (cm)		Leaf width (cm)		Leaf number (No)		Yield (Kg/ha)	
	ADH	75.0	a	130.5	a	59.9	a	27.1	B	19.7	b	3274	a
Line 1	Inbred	75.3	a	133.6	a	58.7	ab	26.4	B	21.4	a	3407	a
	MDH	70.4	a	129.7	a	57.4	b	31.7	A	20	b	3492	a
	ADH	69.9	b	127.4	b	57.8	b	31.7	A	19.5	b	3337	a
Line 2	Inbred	79	a	130.3	ab	59.7	a	27.4	B	21.2	a	3394	a
	MDH	77.5	a	133.7	a	58.9	ab	23.4	C	21	a	3172	a
	ADH	70.7	b	123.4	b	58.2	b	31.4	A	19.7	b	3406	a
Line 3	Inbred	78.5	a	129.9	a	60.1	ab	27.1	B	21.1	a	3206	a
	MDH	72.9	b	133	a	60.6	a	27.9	B	20.4	ab	3308	a
	ADH	69.8	b	126.1	b	56.1	a	30.1	A	19.6	b	3349	a
Line 4	Inbred	78	a	131.5	a	58.5	a	26.4	B	21.8	a	3433	a
	MDH	72.3	b	130.1	ab	57.6	a	30.7	A	19.6	b	3400	a
	ADH	72.8	a	130.5	a	58	a	30.6	A	20.5	a	3470	a
Line 5	Inbred	76.3	a	132.2	a	58.6	a	27	B	21.4	a	3308	a
	MDH	-	-	-	-	-	-	-	-	-	-	-	-
	ADH	71.3	a	129.4	a	60.0	a	27.8	A	19.5	b	3356	a
Line 6	Inbred	76.8	a	132.6	a	59.1	a	27.3	A	21.4	a	3441	a
	MDH	-	-	-	-	-	-	-	-	-	-	-	-
	ADH	76.9	a	132.8	a	63.9	a	21.7	B	21.5	a	3155	a
Line 7	Inbred	76.5	a	129.9	a	58.7	b	26.8	A	20.7	a	3259	a
	MDH	-	-	-	-	-	-	-	-	-	-	-	-
	ADH	74.7	a	130.6	a	58.8	a	27.1	B	21.1	a	3512	a
Line 8	Inbred	72.8	a	129.9	a	60.9	a	28.4	A	20.4	a	3359	a
	MDH	-	-	-	-	-	-	-	-	-	-	-	-

Means followed by the same letter within a column and within a TN 90LC line are not significantly different at the 5% level of significance.

Among the four MDDH lines, lines one and three compared most closely to the inbred GR 149LC source (table 3.29). In comparison to the inbred source, MDDH line one had a significantly lower leaf width and leaf number, while line three was significantly taller at the 50th day after transplant. Neither MDDH line one or three was significantly different from GR 149LC for any other trait; although the differences were not statistically significant, both lines produced slightly higher yields than did GR 149LC. MDDH line two differed significantly from the inbred source only for leaf width. MDDH line four was significantly inferior to GR 149LC for plant height at the 50th day after transplanting and leaf number, but it had a significantly higher number of leaves. Although MDDH lines two and four were slightly lower yielding then GR 149LC, the differences were not statistically significant.

3.3.3 Agronomic Performance of Hybrids derived from Doubled Haploid Lines

KT 204LC family – Location effects

Six agronomic traits were measured for the three populations of the KT 204LC family. The study was carried out at the GR, HR and the WC locations during the 2014 growing season. For all the traits, there were significant differences between locations (table 3.30). The GR location displayed the shortest plants at the 50th day after transplant for all three populations, but at this location the tallest plants after topping for the AD(F_1) and hybrid KT 204LC check population were observed (table 3.31). For the GR and HR locations, the hybrid check was taller than the other two populations at 50 days after transplant and after topping, but the AD(F_1) method was taller at the WC locations (table 3.31).

The length and width of leaves were consistent across locations. For all three populations, the GR location displayed the largest leaves and the HR location the smallest leaves. The AD(F_1) KT 204LC population displayed the longest leaves at the GR and HR locations. At the WC location, the hybrid check had the longest leaves (table 3.31).

Table 3.30 – Analysis of variance of differences among locations for the KT 204LC family.

Statistics	Height 50	Height topping	Leaf length	Leaf width	Leaf number	yield
DF	2	2	2	2	2	2
MS	176.22	468.73	1196.94	332.82	105.2	4961038.58
F value	4.25*	14.24***	182.18***	113.55***	145.14***	52.85***

(*), (**), (***) - Differences between lines significant at P<0.05, 0.01, 0.0001, respectively.

The difference in the width of leaves among all the three methods was 1.4 cm at WC, 0.5 cm at GR, and just 0.1 cm at the HR location. The HR location produced the highest number of leaves per plant for all the three KT 204LC populations, but yielded the least amount of cured leaves/hectare for all three populations (table 3.31).

The MD(F_1) KT 204LC yielded more Kg/ha at the GR and WC locations, while the hybrid KT 204LC check population was superior at the HR location (table 3.31). The WC location was superior to the other two locations for all three methods, with the HR location inferior to the others. The MD(F_1) KT 204LC populations yielded more Kg/ha of cured leaves at the GR and WC locations, and the hybrid check was the best performing at the HR location (table 3.31).

KT 204LC family – Comparison of methods for obtaining DH(F_1) lines

The ANOVA and mean values of methods for obtaining DH(F_1) hybrid lines are presented in Tables 3.32 and 3.33, respectively. For plant height at 50 days after transplanting, there were statistically significant differences between the AD(F_1) and the hybrid check populations, but not between the MD(F_1) and the hybrid KT 204LC check population (table 3.32). On average, the hybrid check KT 204LC was taller than the AD(F_1) (2.4%) and taller than the MD(F_1) KT 204LC populations (1%) (table 3.33). However, since there was a statistically significant “location by line by method” interaction in the comparisons between AD(F_1) and the hybrid check populations (table A 3.2 in the appendix), the significance of the difference between methods is invalidated by the significance of the interaction. The plant height after topping of the check KT 204LC population was 130.3 cm, which was 1.15% taller than the AD(F_1) and 0.9% taller than the MD(F_1) KT 204LC populations (table 3.33); neither of these differences were statistically significant (table 3.32).

The AD(F_1), MD(F_1) and the hybrid check KT 204LC displayed average leaf length of 64 cm, 63.7 cm and 63.1 cm, respectively (table 3.33). The 0.9 cm difference (1.3%) between AD(F_1) KT 204LC and the hybrid check populations was statistically significant, but not the 0.6 cm (0.8%) between

Table 3.31 – Agronomic traits means for the populations of the KT 204LC family by location, 2014.

Trait	Location	---------- Methods ----------		
		AD(F_1)	Hybrid	MD(F_1)
Height 50 (cm)	GR	78.2	82.1	80.8
	HR	80.6	83	82.7
	WC	83.6	83.3	82.5
Height topping (cm)	GR	131.3	133.1	130.7
	HR	126.5	130.5	125.1
	WC	128.4	127.3	131.3
Leaf Length (cm)	GR	64.9	63.4	64.8
	HR	61.1	59.6	61
	WC	62	62.7	61.5
Leaf Width (cm)	GR	30.9	30.4	30.7
	HR	26.5	26.6	26.6
	WC	27.6	28.1	29.0
Number of Leaves per Plant (No)	GR	19.1	19.4	19.3
	HR	21.6	22	21.3
	WC	20.2	20.1	20.7
Yield (kg/ha)	GR	3115	3194	3251
	HR	2947	3027	2956
	WC	3432	3351	3679

Table 3.32 - Analysis of variance of differences among methods to generate DH lines (source of variation) in the KT 204LC family for six agronomic traits.

Comparison	Statistic	Height 50	Height topping	Leaf length	Leaf width	Leaf Number	Yield
Between	DF	2	2	2	2	2	2
all three	MS	93.6	62.56	14.6	3.67	1.40	347838.78
populations	F value	2.26	1.9	2.22	1.25	1.93	3.71*
Inbred	DF	1	1	1	1	1	1
x	MS	184.53	89.16	28.09	0.27	2.34	46966.27
ADDH	F value	4.99*	3.97	4.18*	0.09	3.57	0.61
Inbred	DF	1	1	1	1	1	1
x	MS	28.9	85.12	12.15	3.27	0.97	401571.1
MDDH	F value	0.96	2.64	1.75	1.14	1.32	3.56

(*), (**), (***) - Differences between populations significant at P<0.05, 0.01, 0.0001, respectively.

Table 3.33 - Mean, standard deviation, standard error, coefficient of variation, minimum and maximum values for F_1 hybrid KT 204LC, AD(F_1) and MD(F_1) populations over three locations - 2014 (GR, HR and WC).

Statistics	Height 50 (cm)	Height topping (cm)	Leaf length (cm)	Leaf width (cm)	Leaves per plant	Yield (Kg/ha)
---------- AD(F_1) KT 204LC population ----------						
Mean	80.79	128.77	63.96	28.37	20.27	3195
Std Dev	11.06	9.87	4.51	2.68	1.5	390.4
Std Error	1.17	1.05	0.48	0.29	0.16	43.93
CV	0.14	0.08	0.07	0.09	0.07	0.12
Minimum	48.75	103.67	54	22	17	2212
Maximum	104.5	150	74.83	37.33	25.17	4100
---------- Hybrid KT 204LC population ----------						
Mean	82.81	130.29	63.14	28.45	20.46	3211
Std Dev	9.29	8.9	4.11	2.34	1.6	300.7
Std Error	0.98	0.95	0.44	0.25	0.17	33.62
CV	0.11	0.07	0.07	0.08	0.08	0.09
Minimum	60.13	109.67	53.83	23.83	17.5	2466
Maximum	102.5	148.33	70.83	34	24.33	4043
---------- MD(F_1) KT 204LC population ----------						
Mean	82.01	129.14	63.68	28.83	20.4	3342
Std Dev	10.35	8.85	4.44	2.44	1.24	435.9
Std Error	1.09	0.95	0.48	0.26	0.13	49.04
CV	0.13	0.07	0.07	0.08	0.06	0.13
Minimum	55.38	104.5	52.33	23	17	2394
Maximum	107.38	149.33	71.83	33.83	23	4444

MD(F_1) and the hybrid KT 204LC check populations (table 3.32). The width of leaves for the AD(F_1) KT 204LC population was 28.4 cm, the hybrid check was 28.5 cm, and the MD(F_1) KT 204LC population, 28.8 cm (table 3.33). Those values represent a difference of 0.2% between AD(F_1) and the hybrid check and 1.3% between MD(F_1) and the hybrid check KT 204LC, which were not significantly different (table 3.32). Both the AD(F_1) and the MD(F_1) KT 204LC populations had fewer leaves per plant than the hybrid KT 204LC check population (table 3.33), but there were no statistically significant differences among any of the methods (table 3.32). The ANOVA showed no statistically significant differences among any of the populations for yield (table 3.32). The AD(F_1), the hybrid check and the MD(F_1) KT 204LC populations yielded 3195, 3211 and 3342 Kg/ha, respectively (table 3.33).

KT 204LC family – Comparison of individual lines within methods for obtaining DH(F_1) lines

Surprisingly, the hybrid check KT 204LC was the only population among the three methods which showed statistically differences between individual lines for plant height at the 50th day after transplant (table 3.34). The means separation (Fischer's LSD) for the check population, as well as the mean values for plant height at the 50th day after transplant, ranked from tallest to shortest, for the 30 individual lines included among the hybrid KT 204LC, ADDH and MDDH populations are presented in Table 3.35. The ranks for the ten AD(F_1) lines ranged from 4-29; only two of the lines ranked in the top ten, while six of the lines ranked 20th or higher. The ranks for plant height for the ten MD(F_1) KT 204LC lines ranged from 2-30, with four lines ranking in the top ten and two lines having a rank of twenty or higher.

There were statistically significant differences among individual lines of the MD(F_1) population and among lines of the hybrid check KT 204LC population for plant height after topping, but not among lines within the AD(F_1) population (table 3.34). Mean separation of all methods are presented in Table 3.35. Although not statistically different, the ranks for the ten AD(F_1) lines ranged from 5-29, with three of the lines ranked in the top ten and four of the lines ranked 20th or higher.

Table 3.34 – Analysis of variance for differences between lines (source of variation) within populations, for six agronomic traits.

Population	Statistic	Height 50	Height topping	Leaf length	Leaf width	Leaf number	Yield
AD(F_1) KT 204LC	MS	58.55	99.57	21.41	3.58	2.23	147819.8
	F value	0.72	1.27	2.72*	1	1.91	1.88
hybrid KT 204LC	MS	59.07	88.55	7.96	1.9	0.81	74214.4
	F value	2.31*	2.78**	1.01	0.58	0.81	0.96
MD(F_1) KT 204LC	MS	112.17	144.12	13.27	4.09	0.88	137434.2
	F value	1.47	2.57*	1.72	1.33	1.11	1.14

(*), (**), (***) - Differences between lines within the same population significant at P<0.05, 0.01, 0.0001, respectively.

Degrees of Freedom for all populations of KT 204LC = 9;

Although relative uniformity was expected in the hybrid check KT 204LC population, the ten replications ranged from 1-26; this was likely due to the fact that different workers randomly topped the individual plots. The rank for plant height after topping for the ten MD(F_1) lines ranged from 3-30, with three lines ranking in the top ten and four lines having a rank of 20 or higher (table 3.35).

For leaf length, AD(F_1) KT 204LC was the only population that had statistically significant differences among individual lines (table 3.34), with the lines ranking from 1-30 (table 3.36). None of the populations displayed lines with leaves significantly wider or narrower than other lines within the respective populations (table 3.34). The ranks for the ten AD(F_1) lines ranged from 6-30, with two of the lines ranked in the top ten and three of the lines ranked 20th or higher. The ranks for leaf width for the ten MD(F_1) lines ranged from 1-27, with five lines ranking in the top ten and just two lines having a rank of 20 or higher (table 3.36).

For the number of leaves per plant trait, no statistically significant difference was detected among individual lines within any of the respective populations (table 3.34). Although the differences were not significant, within the AD(F_1) population the ranks of individual lines ranged from 1-30, with three of the lines ranked in the top 10 and five of the lines ranked 20th or higher (table 3.37). The ranks for leaf number for the ten MD(F_1) lines ranged from 3-28, with three lines ranked in the top ten and three lines ranked 20th or higher.

For yield, there were no statistically significant differences among lines of any of the three populations (table 3.34). The ten AD(F_1) KT 204LC iterations ranged in rank from 2-30, with just two lines ranking in the top ten and six ranking 20th or higher (table 3.37). The ranks for the ten MD(F_1) lines ranged from 1-28, with just one line ranked 20th or higher and seven lines ranking in the top ten.

Table 3.35 – Means separation and ordinal ranking of lines by descending order of plant height at the 50th day after transplant and height after topping of the KT 204LC family.

Line	Method	Height 50 (cm)	Rank*	Height topping (cm)	Rank*
1	AD(F_1)	76.2	29th	127.9	20th
2	AD(F_1)	81.5	19th	130.6	11th
3	AD(F_1)	81	21st	131.7	10th
4	AD(F_1)	80.8	23rd	132.1	8th
5	AD(F_1)	84.3	7th	133.2	5th
6	AD(F_1)	78.6	26th	126.3	27th
7	AD(F_1)	78.5	27th	122.5	29th
8	AD(F_1)	81.5	17th	128.2	19th
9	AD(F_1)	84.5	4th	125.7	28th
10	AD(F_1)	80.9	22nd	129	17th
1	Hybrid	84 ab	8th	135.1 a	1st
2	Hybrid	83.1 ab	13th	134.8 ab	2nd
3	Hybrid	84.5 ab	5th	129 cde	16th
4	Hybrid	87 a	1st	132.4 abc	6th
5	Hybrid	79.7 bc	24th	129.6 abcd	14th
6	Hybrid	83.2 ab	11th	132.2 abcd	7th
7	Hybrid	81.2 bc	20th	127.5 cde	22nd
8	Hybrid	84.4 ab	6th	126.8 de	25th
9	Hybrid	83.1 ab	12th	128.9 cde	18th
10	Hybrid	78.1 c	28th	126.6 e	26th
1	MD(F_1)	86.1	2nd	130.5 a	12th
2	MD(F_1)	83.4	10th	133.4 a	3rd
3	MD(F_1)	84	9th	133.3 a	4th
4	MD(F_1)	85.6	3rd	129.6 a	15th
5	MD(F_1)	74	30th	119.8 b	30th
6	MD(F_1)	82.3	15th	127.5 a	23rd
7	MD(F_1)	81.6	18th	127.4 a	24th
8	MD(F_1)	82.6	14th	129.8 a	13th
9	MD(F_1)	82	16th	127.6 a	21st
10	MD(F_1)	78.6	25th	131.9 a	9th
	Range	13		15.4	

(*) Rank includes all three populations, not discriminated by method.

Means within columns of the same method followed by the same letter are not different at the 5% level of significance, based on Fischer's LSD.

Height 50 - Hybrid method: Critical value of t = 2 and LSD = 4.7816.

Height topping – Hybrid method: Critical value of t = 2.008 and LSD = 5.438.

MDH method: Critical value of t = 2.008 and LSD = 5.675.

Table 3.36 – Means separation and ordinal ranking by descending order of leaf length and leaf width of populations of the KT 204LC families.

Line	Method	Leaf length (cm)	Rank*	Leaf width (cm)	Rank*
1	AD(F_1)	63.9 ab	14th	28.8	10th
2	AD(F_1)	64.2 ab	8th	28.8	12th
3	AD(F_1)	64 ab	12th	28.6	15th
4	AD(F_1)	64.1 ab	9th	28.5	18th
5	AD(F_1)	60.4 c	30th	27	30th
6	AD(F_1)	65.1 ab	3rd	28.1	25th
7	AD(F_1)	66.4 a	1st	29.1	6th
8	AD(F_1)	63.1 b	20th	27.6	29th
9	AD(F_1)	64.4 ab	5th	28.5	17th
10	AD(F_1)	64.1 ab	10th	28.8	11th
1	Hybrid	63.4	19th	28.8	9th
2	Hybrid	63.1	21st	28.7	13th
3	Hybrid	61.3	29th	27.8	28th
4	Hybrid	63.5	18th	28.3	22nd
5	Hybrid	62	28th	28.2	23rd
6	Hybrid	64.4	6th	29.2	5th
7	Hybrid	63	22nd	28.3	20th
8	Hybrid	62.7	25th	28.1	24th
9	Hybrid	63.9	15th	28.3	21st
10	Hybrid	64.2	7th	28.9	8th
1	MD(F_1)	63.8	17th	29.3	3rd
2	MD(F_1)	64	13th	29.3	4th
3	MD(F_1)	63	23rd	29	7th
4	MD(F_1)	63.8	16th	28.5	16th
5	MD(F_1)	64.8	4th	27.9	26th
6	MD(F_1)	62.8	24th	27.8	27th
7	MD(F_1)	62.3	27th	28.7	14th
8	MD(F_1)	62.3	26th	28.3	19th
9	MD(F_1)	64.1	11th	29.6	2nd
10	MD(F_1)	66.1	2nd	29.8	1st
	Range	6		2.9	

(*) Rank includes all three populations, not discriminated by method.

Means within columns of the same method followed by the same letter are not different at the 5% level of significance, based on Fischer's LSD.

Leaf length - ADH method: Critical value of t = 2.007 and LSD = 2.6862.

Table 3.37 - Means separation and ordinal ranking by descending order of number of leaves per plant and yield for populations of the KT 204LC family.

Line	Method	Leaves per plant	Rank*	Yield (Kg/ha)	Rank*
1	AD(F1)	19.9	27th	3317	13th
2	AD(F1)	20.5	10th	3350	9th
3	AD(F1)	20.7	9th	3094	27th
4	AD(F1)	20.4	13th	3101	26th
5	AD(F1)	21.4	1st	3256	15th
6	AD(F1)	20.1	23rd	3437	2nd
7	AD(F1)	19.7	29th	3145	22nd
8	AD(F1)	20.1	24th	3135	23rd
9	AD(F1)	19.4	30th	3022	30th
10	AD(F1)	20.4	14th	3108	25th
1	Hybrid	21	2nd	3159	20th
2	Hybrid	20.8	5th	3276	14th
3	Hybrid	20.4	12th	3377	8th
4	Hybrid	20.4	17th	3119	24th
5	Hybrid	20.7	8th	3186	18th
6	Hybrid	20.7	6th	3331	11th
7	Hybrid	20.4	16th	3237	16th
8	Hybrid	20.1	22nd	3152	21st
9	Hybrid	20	25th	3223	17th
10	Hybrid	20.2	21st	3052	29th
1	MD(F1)	20.2	20th	3489	1st
2	MD(F1)	20.9	3rd	3396	5th
3	MD(F1)	20.9	4th	3318	12th
4	MD(F1)	20.4	15th	3422	4th
5	MD(F1)	19.8	28th	3390	6th
6	MD(F1)	20.3	18th	3175	19th
7	MD(F1)	20	26th	3078	28th
8	MD(F1)	20.7	7th	3389	7th
9	MD(F1)	20.3	19th	3431	3rd
10	MD(F1)	20.5	11th	3344	10th
	range	2		466	

KT 204LC family – Direct comparison of each AD(F_1) and MD(F_1) line with the hybrid check

The statistics for the independent analysis for the KT 204LC family are listed in Tables 3.38 - 3.40. Location X method interactions were detected for only ten of 60 total observations. Interactions were especially present for two lines; significant differences were detected for triplet five for the traits height 50, height topping, leaf length, leaf number and yield, and for triplet ten for height 50, height topping, and leaf number. Otherwise, significant location X method interactions were detected only for plant height after topping for triplet three and number of leaves per plant for triplet four. The KT 204LC AD(F_1) and MD(F_1) hybrid lines in triplet five were originated from the TN 90LC ADDH line five and MDDH line five, respectively, both of which were visibly off-type. Even though the mean yields of those DH(F_1) KT 204LC lines five (table 3.41) were higher compared to the TN 90LC DH parental line, considerable variation was observed in the hybrids.

When the data were pooled across locations, for the AD(F_1) method seven of the ten lines (2, 4, 5, 6, 8, 9, and 10) did not differ significantly from the KT 204LC check for any trait measured (table 3.41). In comparison to the KT 204LC check, only AD(F_1) line one was significant different for any trait, having shorter plant height and a lower leaf number. Conversely, AD(F_1) line three was significant different to the check for leaf length, and AD(F_1) line seven was statistically significant different for leaf length. Although differences were not significant, lines 1, 2, 5, 6, and 10 produced yields that were equal to or higher than the check. For the MD(F_1) method, six of the ten lines (2, 6, 7, 8, 9, and 10) did not differ significantly from the KT 204LC check for any trait measured (table 3.41). MD(F_1) line five was statistically different to the hybrid check KT 204LC for the traits plant height at the 50th day after transplant, plant height after topping and for leaf length (table 3.41). As mentioned earlier, the MDDH pollinator for this hybrid was substantially off-type and significant location by method interactions were detected in triplet five for all traits except leaf width.

Table 3.38 - Analysis of variance of differences between methods within the lines of the KT 204LC population, for the agronomic traits plant height at the 50th day after transplant and plant height after topping.

Triplets	statistics	Height 50 ---------- Source of variation ---------				Height topping --------- Source of variation ----------			
		loc	rep(loc)	method	loc x method	loc	rep(loc)	method	loc x method
Line 1	DF	2	6	2	4	2	6	2	4
	MS	106.07	256.52	245.66	17.86	13.79	217.22	54.39	42.68
	F Value	3.57	8.63**	8.26**	0.6	0.69	10.83**	2.71	2.13
Line 2	DF	2	6	2	4	2	6	2	4
	MS	5.62	345.10	9.51	51.64	50.39	105.68	38.90	39.48
	F Value	0.18	10.8**	0.30	1.62	2.42	5.07**	1.87	1.89
Line 3	DF	2	6	2	4	2	6	2	4
	MS	186.69	207.03	31.24	7.84	38.18	108.11	41.17	96.87
	F Value	3.84	4.26*	0.64	0.16	1.81	5.13**	1.95	4.6*
Line 4	DF	2	6	2	4	2	6	2	4
	MS	43.02	360.45	95.49	28.60	90.55	258.64	21.60	60.45
	F Value	0.91	7.59**	2.01	0.6	4.51*	12.87**	1.08	3.01
Line 5	DF	2	6	2	4	2	6	2	4
	MS	251.87	153.08	241.92	90.16	112.86	38.26	544.66	280.02
	F Value	10.49**	6.37**	10.07**	3.75*	5.13*	1.74	24.78***	12.74***
Line 6	DF	2	6	2	4	2	6	2	4
	MS	51.32	362.89	53.18	20.53	194.93	314.15	88.11	41.41
	F Value	1.09	7.68**	1.13	0.43	4.08*	6.57**	1.84	0.87
Line 7	DF	2	6	2	4	2	6	2	4
	MS	31.18	206.94	23.79	67.19	63.03	91.88	86.74	27.18
	F Value	0.33	2.19	0.25	0.71	0.80	1.17	1.11	0.35
Line 8	DF	2	6	2	4	2	6	2	4
	MS	232.30	397.63	18.34	37.62	259.78	180.65	19.94	42.77
	F Value	6.06*	10.37**	0.48	0.98	7.84**	5.45**	0.60	1.29
Line 9	DF	2	6	2	4	2	6	2	4
	MS	25.50	218.66	13.80	52.47	322.98	100.46	31.83	32.37
	F Value	0.95	8.11**	0.51	1.95	10.65**	3.31*	1.05	1.07
Line 10	DF	2	6	2	4	2	6	2	4
	MS	284.07	182.52	20.72	248.47	346.90	123.70	63.76	156.57
	F Value	10.88**	6.99**	0.79	9.52**	9.01**	3.21*	1.66	4.07*

(*), (**), (***) - Differences between lines within the same triplet significant at P<0.05, 0.01, 0.0001, respectively.

Table 3.39 - Analysis of variance of differences between methods within the lines of the KT 204LC population, for the agronomic traits leaf length and leaf width.

Triplets	statistics	Leaf Length --------- Source of variation ---------				Leaf Width ---------- Source of variation ----------			
		loc	rep(loc)	method	loc x method	loc	rep(loc)	method	loc x method
	DF	2	6	2	4	2	6	2	4
Line 1	MS	144.03	6.06	0.49	8.56	23.01	1.91	1.40	3.80
	F Value	17.99**	0.76	0.06	1.07	11.09**	0.92	0.67	1.83
	DF	2	6	2	4	2	6	2	4
Line 2	MS	77.88	6.68	3.13	0.44	18.26	2.10	0.11	1.98
	F Value	11**	0.94	0.44	0.06	5.36*	0.62	0.03	0.58
	DF	2	6	2	4	2	6	2	4
Line 3	MS	266.45	8.84	16.62	6.80	74.22	2.16	3.79	2.13
	F Value	52.57***	1.75	3.28	1.34	77.32***	2.25	3.94*	2.22
	DF	2	6	2	4	2	6	2	4
Line 4	MS	114.57	10.54	0.80	3.33	21.19	3.82	0.19	1.29
	F Value	19.13**	1.76	0.13	0.56	4.38*	0.79	0.04	0.27
	DF	2	6	2	4	2	6	2	4
Line 5	MS	92.09	6.89	40.94	16.81	35.32	5.80	3.53	4.74
	F Value	22.43***	1.68	9.97**	4.09*	23.21***	3.81*	2.32	3.11
	DF	2	6	2	4	2	6	2	4
Line 6	MS	149.18	6.58	12.54	7.84	43.82	4.30	4.60	1.04
	F Value	30.76***	1.36	2.59	1.62	17.71**	1.74	1.86	0.42
	DF	2	6	2	4	2	6	2	4
Line 7	MS	118.12	7.92	39.92	13.24	46.63	2.63	2.65	11.84
	F Value	12.56**	0.84	4.24*	1.41	10.69**	0.60	0.61	2.72
	DF	2	6	2	4	2	6	2	4
Line 8	MS	137.52	10.75	1.28	16.89	42.94	5.40	1.19	1.56
	F Value	13.54***	1.06	0.13	1.66	17.74**	2.23	0.49	0.65
	DF	2	6	2	4	2	6	2	4
Line 9	MS	186.41	11.34	0.74	2.86	35.86	8.37	2.81	0.77
	F Value	58.26***	3.54*	0.23	0.89	9.22**	2.15	0.72	0.20
	DF	2	6	2	4	2	6	2	4
Line 10	MS	101.51	20.23	11.86	7.40	52.68	2.97	2.92	1.94
	F Value	12.96**	2.58	1.51	0.95	14.82**	0.84	0.82	0.54

(*), (**), (***) - Differences between lines within the same triplet significant at P<0.05, 0.01, 0.0001, respectively.

Table 3.40 - Analysis of variance of differences between methods within the lines of the KT 204LC population, for the agronomic traits number of leaves per plant and yield.

Triplets	statistics	Leaf Number --- Source of variation ---				Yield --- Source of variation ---			
		loc	rep(loc)	method	loc x method	loc	rep(loc)	method	loc x method
Line 1	DF	2	6	2	4	2	5	2	4
	MS	7.85	1.27	2.23	0.39	476018.7	86925.8	239000.7	58966.7
	F Value	22.93**	3.7*	6.51*	1.13	2.77	0.51	1.39	0.34
Line 2	DF	2	6	2	4	2	5	2	4
	MS	8.58	1.01	0.38	0.80	180086.5	68152.4	21788.9	171753.4
	F Value	14.6	1.73	0.65	1.36	2.74	1.04	0.33	2.62
Line 3	DF	2	6	2	4	2	5	2	4
	MS	17.09	1.30	0.41	1.25	551085.7	91077.0	133005.1	91687.6
	F Value	34.12	2.6	0.81	2.49	6.96*	1.15	1.68	1.16
Line 4	DF	2	6	2	4	2	5	2	4
	MS	13.34	1.15	0.01	1.95	907167.6	109286.8	244144	161655
	F Value	52.35***	4.53*	0.03	7.67**	18.68**	2.25	5.03*	3.33
Line 5	DF	2	6	2	4	2	5	2	4
	MS	16.99	1.74	6.50	4.59	657508.3	191209.2	54250.5	432240.2
	F Value	21.93***	2.24	8.39**	5.92**	10.22**	2.97	0.84	6.72**
Line 6	DF	2	6	2	4	2	5	2	4
	MS	10.56	3.92	0.96	1.08	341623.9	64937.8	201126.9	108704.4
	F Value	9.06**	3.37*	0.82	0.92	2.18	0.41	1.28	0.69
Line 7	DF	2	6	2	4	2	5	2	4
	MS	12.15	0.80	2.28	0.93	236451.5	46214.3	69820.2	126502.4
	F Value	8.05**	0.53	1.51	0.62	2.58	0.51	0.76	1.38
Line 8	DF	2	6	2	4	2	5	2	4
	MS	12.81	3.29	1.09	0.52	1089794.9	156320.1	148077.2	54115.8
	F Value	14.73**	3.79*	1.25	0.60	12.6**	1.81	1.71	0.63
Line 9	DF	2	6	2	4	2	5	2	4
	MS	6.96	0.42	1.41	1.38	670832.7	164447.5	365874.3	65890.7
	F Value	12.19**	0.74	2.46	2.41	11.95**	2.93	6.52*	1.17
Line 10	DF	2	6	2	4	2	5	2	4
	MS	16.14	1.11	0.22	3.70	461287.0	88478.4	184804.5	78083.4
	F Value	22.77***	1.57	0.31	5.21*	3.74	0.72	1.50	0.63

(*), (**), (***) - Differences between lines within the same triplet significant at P<0.05, 0.01, 0.0001, respectively.

Table 3.41 – Agronomic trait means for lines of the KT 204LC family and separation of means within triplets based on Fischer's LSD.

KT 204LC triplet	Method	Height 50 (cm)		Height topping (cm)		Leaf length (cm)		Leaf width (cm)		Leaf number (No)		Yield (kg/ha)	
1	AD(F_1)	76.2	b	127.9	b	64.0	a	28.8	a	19.9	b	3317	a
	Hybrid	84.0	a	135.1	a	63.4	a	28.8	a	21.0	a	3159	a
	MD(F_1)	86.1	a	130.5	ab	63.8	a	29.3	a	20.2	b	3489	a
2	AD(F_1)	81.5	a	130.6	a	64.2	a	28.8	a	20.5	a	3350	a
	Hybrid	83.1	a	134.8	a	63.0	a	28.7	a	20.8	a	3276	a
	MD(F_1)	83.4	a	133.4	a	64.0	a	29.3	a	20.9	a	3396	a
3	AD(F_1)	81	a	131.7	a	64	a	28.6	ab	20.7	a	3094	a
	Hybrid	84.5	a	129	a	61.3	b	27.8	b	20.4	a	3377	a
	MD(F_1)	84	a	133.3	a	63.0	ab	29	a	20.9	a	3318	a
4	AD(F_1)	80.8	a	132.1	a	64.1	a	28.5	a	20.4	a	3101	b
	Hybrid	87	a	132.4	a	63.5	a	28.3	a	20.4	a	3119	b
	MD(F_1)	85.6	a	129.6	a	63.8	a	28.5	a	20.4	a	3422	a
5	AD(F_1)	84.3	a	133.2	a	60.4	b	27	a	21.4	a	3256	a
	Hybrid	79.7	a	129.6	a	62	b	28.2	a	20.7	ab	3186	a
	MD(F_1)	74	b	119.8	b	64.7	a	27.9	a	19.8	b	3390	a
6	AD(F_1)	78.6	a	126.3	a	65.1	a	28.1	a	20.1	a	3437	a
	Hybrid	83.2	a	132.2	a	64.4	ab	29.1	a	20.7	a	3331	a
	MD(F_1)	82.3	a	127.5	a	62.8	b	27.8	a	20.3	a	3175	a
7	AD(F_1)	78.5	a	122.5	a	66.4	a	29.1	a	19.7	a	3145	a
	Hybrid	81.2	a	127.5	a	63	b	28.7	a	20.4	a	3237	a
	MD(F_1)	81.5	a	127.4	a	62.3	b	28.3	a	20	a	3078	a
8	AD(F_1)	81.5	a	128.2	a	63.1	a	27.6	a	20.1	a	3135	a
	Hybrid	84.4	a	126.8	a	62.7	a	28.1	a	20.1	a	3152	a
	MD(F_1)	82.6	a	129.8	a	62.3	a	28.3	a	20.7	a	3389	a
9	AD(F_1)	84.5	a	125.7	a	64.4	a	28.5	a	19.4	b	3022	b
	Hybrid	83.1	a	128.9	a	63.9	a	28.3	a	20	ab	3223	ab
	MD(F_1)	82	a	127.6	a	64.1	a	29.6	a	20.3	a	3431	a
10	AD(F_1)	80.9	a	129	a	64.1	a	28.8	a	20.4	a	3108	a
	Hybrid	78.1	a	126.6	a	64.2	a	28.9	a	20.2	a	3052	a
	MD(F_1)	78.6	a	131.9	a	66.1	a	29.8	a	20.5	a	3344	a

Means followed by the same letter within a column and within a KT 204LC line are not significantly different at the 5% level of significance.

It is interesting to note that the ADDH line five and the MDDH line five parental lines produced 1371 and 1584 Kg/ha less than the TN 90LC inbred source, respectively, (table 3.17), but the hybrids using those DH lines as pollinators, KT 204LC AD(F_1) line five and MD(F_1) line five, were both superior to the hybrid check in terms of yield. In comparison to the KT 204LC check, MD(F_1) line one was inferior for leaf number, while MD(F_1) line five was superior for leaf length and line four was significant for yield. As was observed for the ADDH method, none of the MD(F_1) lines were significantly lower yielding than the KT 204LC check; in fact, all MD(F_1) lines except three, six, and seven produced mean yields higher than the check, but the difference was significant only for line four.

TN 97LC family – Location effects

As observed in the trials of the TN 90LC, GR 149LC and KT 204LC families, there was also a significant difference between the three locations for all six traits measured in the TN 97LC experiment (table 3.42). In the comparison between populations grown at the same location, the hybrid TN 97LC check population was taller than both AD(F_1) and MD(F_1) TN 97LC populations (height 50 and height topping). The hybrid check also produced the highest number of leaves per plant at all locations (table 3.43). The AD(F_1) method was superior to the other methods for the leaf width trait at all locations, leaf length at the HR and WC locations and yield at the GR and WC locations. The MD(F_1) TN 97LC population was superior to the other methods only for the leaf length at the GR location and the yield trait at the HR location (table 3.43). The GR location was superior to the WC and HR locations for the traits height after topping, leaf length, leaf width and yield. The HR location was inferior to the other two locations for all traits measured in all populations (except for leaf number of the hybrid check) (table 3.43).

Table 3.42 – Analysis of variance of differences among locations for the TN 97LC family.

Statistics	Height 50	Height topping	Leaf length	Leaf width	Leaf number	Yield
DF	2	2	2	2	2	2
MS	243.76	1480.67	2831.50	456.34	73.77	5217516.68
F value	6.69**	34.82***	403.75***	145.15***	49.85***	97.78***

(*), (**), (***) - Differences significant at P<0.05, 0.01, 0.0001, respectively.

Table 3.43 – Agronomic traits means for hybrids TN 97LC and respective AD(F_1) and MD(F_1) populations by locations.

Trait	Location	---------- Methods ----------		
		AD(F_1)	Hybrid	MD(F_1)
Height 50 (cm)	GR	79.3	83.5	78.2
	HR	77.2	79.6	76.7
	WC	80.3	86.2	77.8
Height topping (cm)	GR	126.1	127.4	126
	HR	114.9	119.4	117.9
	WC	120.9	125.5	123.2
Leaf Length (cm)	GR	66.9	67	67.5
	HR	55	53.4	54.7
	WC	61.5	59.2	60.3
Leaf Width (cm)	GR	31.3	29.9	30.8
	HR	26	24.7	25.8
	WC	28.4	26.6	28.1
Number of Leaves per Plant (No)	GR	19.2	19.5	19.1
	HR	18.8	19.6	19
	WC	20.9	21.2	20.8
Yield (kg/ha)	GR	3217	3134	3194
	HR	2667	2658	2738
	WC	3214	3113	3099

TN 97LC family – Comparison of methods for obtaining DH(F_1) lines

The ANOVA and mean values for methods for obtaining DH(F1) hybrid lines are presented in Tables 3.44 and 3.45, respectively. For plant height at the 50th day after transplant, the AD(F_1) was 5.0% and the MD(F_1) was 6.6% shorter than the hybrid TN 97LC check population (table 3.45); both these differences were statistically significant (table 3.44). The differences in plant height persisted after topping, with AD(F_1) being 3.5cm and MD(F_1) 1.7cm shorter than the TN 97LC hybrid check; again, both differences were statistically significant.

The leaves of AD(F_1) and MD(F_1) TN 97LC populations were 2% and 1.5% longer than the leaves of the hybrid TN 97LC check population, respectively (table 3.45). The difference for leaf length in comparison to the check was significant for the AD(F_1) population, but not for the MD(F_1) population. The hybrid check, the AD(F_1) and the MD(F_1) TN 97LC populations had leaves 27.1 cm, 28.6 cm and 28.3 cm wide. The differences between the methods were statistically significant (table 3.44), and represent a difference of 5.5% and 4.4% between the hybrid check and the AD(F_1) and between MD(F_1) and the check population, respectively.

There were statistically significant differences between both DH-derived populations and the hybrid check population for leaf number (table 3.44). The number of leaves per plant of the AD(F_1), MD(F_1) and the hybrid check populations of TN 97LC were 19.6, 19.7 and 20.1, respectively (table 3.45). No statistically significant differences for yield were detected among the hybrid TN 97LC check population and the AD(F_1) and the MD(F_1) TN 97LC populations (table 3.44). The AD(F_1), hybrid check and MD(F_1) TN 97LC populations produced 3033, 2968 and 3010 Kg/ha of cured leaves, respectively (table 3.45).

Table 3.44 - Analysis of variance of differences among methods (source of variation) of all traits recorded in the TN 97LC family.

Comparison	Statistics	Height 50	Height topping	Leaf length	Leaf width	Leaf Number	Yield
Between	DF	2	2	2	2	2	2
All three	MS	496.83	241.35	35.28	39.24	6.41	80499.9
populations	F value	13.63***	5.68**	5.03**	12.48***	4.33*	1.51
Hybrid	DF	1	1	1	1	1	1
x	MS	617.8	432.87	53.78	78.28	8.27	149377
AD(F1)	F value	21.91***	11.02**	8.93**	31.86***	5.75*	2.88
Hybrid	DF	1	1	1	1	1	1
x	MS	738.1	7.49	25.46	28.17	10.95	1737.5
MD(F1)	F value	15.94**	4.78*	3.39	8.84**	6.73*	0.03

(*), (**), (***) - Differences between populations significant at P<0.05, 0.01, 0.0001, respectively.

Table 3.45 - Mean, standard deviation, standard error, coefficient of variation, minimum and maximum values for the F_1 hybrid TN 97LC and AD(F_1) and MD(F_1) populations over three locations - 2014 (GR, HR and WC).

Statistics	Height 50 (cm)	Height topping (cm)	Leaf length (cm)	Leaf width (cm)	Leaves per plant	Yield (Kg/ha)
---------- AD(F_1) TN 97LC population ----------						
Mean	78.95	120.63	61.11	28.55	19.62	3033
Std Dev	8.69	11.38	5.81	2.97	2.07	391.21
Std Error	1.02	1.34	0.68	0.35	0.24	46.1
CV	0.11	0.09	0.1	0.1	0.11	0.13
Min	60.38	86	47.33	19.67	13.67	2060
Max	97.25	141	71.33	35.5	23.5	4044
---------- Hybrid TN 97LC population ----------						
Mean	83.09	124.1	59.89	27.07	20.1	2968
Std Dev	10.14	8.8	6.04	2.41	1.69	327.13
Std Error	1.19	1.04	0.71	0.28	0.2	38.55
CV	0.12	0.07	0.1	0.09	0.08	0.11
Min	59.88	104.83	50.33	22.83	16	2172
Max	112.75	141.17	72.67	32.5	23.83	3578
---------- MD(F_1) TN 97LC population ----------						
Mean	77.59	122.38	60.81	28.25	19.66	3010
Std Dev	9.02	10.19	6.1	3.08	1.86	316.45
Std Error	1.06	1.2	0.72	0.36	0.22	37.29
CV	0.12	0.08	0.1	0.11	0.09	0.105
Min	58.88	99.83	46.17	21.33	14.17	2194
Max	102.5	144	72.67	35.17	23.00	3691

TN 97LC family – Comparison of individual lines within methods for obtaining DH(F_1) lines

The ANOVA for the differences between individual lines within the three TN 97LC populations is presented in Table 3.46. Lines within each of the three populations were not statistically different for plant height at the 50th day after transplant or height after topping. The plant height 50th day after transplant ranks for the eight AD(F_1) lines ranged from 8-20, with three of the lines ranked in the top ten and two of the lines ranked 15th or higher (table 3.47). The ranks for height 50 for the four MD(F_1) lines ranged from 15-19, with all lines having a rank of 15 or higher. For plant height after topping, the ranks for the eight AD(F_1) lines ranged from 4-20, with just two of the lines ranked in the top ten and five of the lines ranked 15th or higher. The ranks for the four lines of the MD(F1) population ranged from 6-16, with two lines among the top ten and just one having a rank of 15 or higher (table 3.47).

There were also no statistically significant differences in leaf length between lines of the three populations (table 3.46). The ranks for leaf length for the eight AD(F_1) lines ranged from 1-14, with six of the lines ranked in the top ten and no lines ranked 15th or higher. The ranks the four MD(F_1) lines ranged from 2-17, with three lines ranking in the top ten and one line ranked 15th or higher (table 3.48). MD(F_1) TN 97LC was the only population displaying significant differences among the individual lines for leaf width (table 3.46). The ranks for the eight AD(F_1) lines ranged from 2-14, with seven of the lines ranked in the top ten. The ranks for leaf width for the four MD(F_1) lines ranged from 1-20, with three lines ranking in the top ten and one line ranked 20th (table 3.48).

Lines of all three populations were considerably homogeneous for leaf number and yield, with no significant differences detected among individual lines within a specific population (table 3.46). The leaf number ranks for the eight AD(F_1) lines ranged from 2-20, with two of the lines ranked in the top ten and four of the lines ranked 15th or higher. The ranks for the four MD(F_1) lines ranged from 4-19, with just one line ranking in the top ten and two lines having a rank of 15 or higher (table 3.49). The yield of the eight AD(F_1) TN 97LC

Table 3.46 – Analysis of variance for differences between lines (source of variation) within populations, for six agronomic traits.

Population	Statistic	Height 50	Height topping	Leaf length	Leaf width	Leaf number	Yield
AD(F_1) TN 97LC	MS	45.39	64.33	8.50	6.04	1.74	67314.9
	F value	0.71	0.57	0.76	1.37	0.74	0.77
hybrid TN 97LC	MS	27.56	38.12	1.73	0.34	1.57	44094.6
	F value	0.49	0.86	0.33	0.22	1.30	0.84
MD(F_1) TN 97LC	MS	25.25	22.37	19.56	40.48	3.52	72226.6
	F value	0.47	0.35	2.39	10.93***	1.95	1.34

(*), (**), (***) - Differences between lines within the same population significant at P<0.05, 0.01, 0.0001, respectively)

Degrees of Freedom for AD(F_1) and hybrid TN 97LC = 7;

Degrees of Freedom for MD(F_1) TN 97LC population = 3;

Table 3.47 - Ordinal ranking of the lines of the TN 97LC family by descending order of plant height at the 50th day after transplant and plant height after topping.

Line	Method	Height 50 (cm)	Rank*	Height topping (cm)	Rank*
1	AD(F_1)	80.1	12th	124.4	4th
2	AD(F_1)	74.6	20th	117.6	20th
3	AD(F_1)	80.8	8th	121.2	15th
4	AD(F_1)	78.6	14th	124.1	5th
5	AD(F_1)	76.7	18th	117.7	19th
6	AD(F_1)	79.6	13th	119	18th
7	AD(F_1)	80.7	9th	122	14th
8	AD(F_1)	80.6	10th	119.2	17th
1	Hybrid	80.3	11th	127.3	1st
2	Hybrid	83.7	4th	122	13th
3	Hybrid	85.7	1st	126.7	2nd
4	Hybrid	82.9	5th	125.3	3rd
5	Hybrid	84.4	2nd	123.1	9th
6	Hybrid	82.3	6th	123.2	7th
7	Hybrid	81.4	7th	122	12th
8	Hybrid	84.2	3rd	123.2	8th
1	MD(F_1)	78.2	16th	123.5	6th
2	MD(F_1)	75.9	19th	122.8	10th
3	MD(F_1)	78.5	15th	122.4	11th
4	MD(F_1)	77.8	17th	120.9	16th
	range	11.1		9.6	

(*) Rank includes all three populations, not discriminated by method.

Means within columns of the same method followed by the same letter are not different at the 5% level of significance, based on Fischer's LSD.

Table 3.48 – Means separation and ordinal ranking of lines by descending order of leaf length and leaf width of the populations of the TN 97LC family.

Line	method	Leaf length (cm)	Rank*	Leaf width (cm)	Rank*
1	AD(F_1)	60.3	13th	29.3	3rd
2	AD(F_1)	60.0	14th	28.8	6th
3	AD(F_1)	61.0	5th	28.9	4th
4	AD(F_1)	60.7	7th	29.6	2nd
5	AD(F_1)	62.9	1st	28.1	8th
6	AD(F_1)	61.8	3rd	28.6	7th
7	AD(F_1)	60.5	9th	27.1	14th
8	AD(F_1)	61.8	4th	27.8	10th
1	Hybrid	59.5	19th	27.4	11th
2	Hybrid	59.9	15th	26.8	19th
3	Hybrid	60.5	10th	27.3	12th
4	Hybrid	59.3	20th	27.2	13th
5	Hybrid	60.4	11th	27.1	15th
6	Hybrid	59.5	18th	26.9	17th
7	Hybrid	60.3	12th	26.9	18th
8	Hybrid	59.8	16th	27	16th
1	MD(F_1)	59.7	17th	28.9 ab	5th
2	MD(F_1)	60.7	8th	26.3 c	20th
3	MD(F_1)	62.2	2nd	28 b	9th
4	MD(F_1)	60.7	6th	29.8 a	1st
	range	3.6		29.3	3rd

(*) Rank includes all three populations, not discriminated by method.

Means within columns of the same method followed by the same letter are not different at the 5% level of significance, based on Fischer's LSD.

Leaf width - MDH: Critical value of t = 2.005 and LSD = 1.286

Iterations ranged in rank from 1-19, with five lines ranking in the top ten and two ranking 15th or higher. The ranks for the four MD(F_1) lines ranged from 1-28, with just one line ranked 20th or higher and seven lines ranking in the top ten (table 3.49).

TN 97LC family – Direct comparison of each AD(F_1) and MD(F_1) line with the hybrid check

As described earlier for the TN 90LC, GR 149LC, and KT 204LC families, the data for methods to produce DH(F_1) lines of TN 97LC were pooled across locations, because location X method interactions were statistically significant only for height 50 in triplet eight, height topping in triplet six and for yield in triplet two (tables 3.50 - 3.52). Among the AD(F_1) lines, only one entry (triplet seven) did not differ significantly from the TN 97LC check for any of the six traits that were measured. In general, the AD(F_1) lines tended to be shorter than their respective TN 97LC check; these differences in plant height were statistically significant for plant height at the 50th day after transplant for triplets two, three, five and eight, and for plant height after topping for triplets three and six (table 3.53). All eight AD(F_1) lines had leaves that were longer and wider than their TN 97LC checks; however the differences in leaf size were statistically significant only for leaf width in triplets one, two, three and four. Most importantly, none of the eight AD(F_1) lines differed significantly from its respective TN 97LC hybrid check for leaf number or for yield (table 3.53). In fact, six of the eight AD(F_1) lines produced yields that were slightly higher than their respective check.

Among the MD(F_1) lines, all of the four entries differed significantly from their respective TN 97LC check for at least one trait (table 3.53). However, as was seen for the AD(F_1) lines, most of the differences were for plant height or leaf width. All four lines tended to be shorter than their checks, particularly at 50 days after transplanting, where significant differences were detected in triplets two and three. Three of the four MD(F_1) lines produced wider leaves than their checks, but the differences were statistically significant only for triplets one and four In triplet four, the MD(F_1) line had significantly fewer

Table 3.49 - Means separation and ordinal ranking by descending order of the number of leaves per plant and yield of the TN 97LC family.

Line	Method	Leaves per plant	Rank*	Yield	Rank*
1	AD(F_1)	19.4	17th	3035	7th
2	AD(F_1)	19.6	14th	2944	16th
3	AD(F_1)	19.2	18th	3139	1st
4	AD(F_1)	20.5	2nd	3101	3rd
5	AD(F_1)	19	20th	2919	19th
6	AD(F_1)	19.5	16th	2952	14th
7	AD(F_1)	19.7	11th	3128	2nd
8	AD(F_1)	19.9	8th	3043	6th
1	Hybrid	20.1	6th	3003	11th
2	Hybrid	20.1	5th	2953	13th
3	Hybrid	20.1	7th	3034	8th
4	Hybrid	21	1st	3018	10th
5	Hybrid	19.7	10th	2948	16th
6	Hybrid	19.8	9th	2938	18th
7	Hybrid	19.7	13th	2823	20th
8	Hybrid	20.3	3rd	3029	9th
1	MD(F_1)	19.6	15th	3078	4th
2	MD(F_1)	20.2	4th	2964	12th
3	MD(F_1)	19.1	19th	2950	15th
4	MD(F_1)	19.7	12th	3050	5th
	range	2		316	

(*) Rank includes all three populations, not discriminated by method.

Table 3.50 - Analysis of variance of differences between methods within the lines of the TN 97LC population, for the agronomic traits plant height at the 50th day after transplant and plant height after topping.

TN 97LC Triplet	statistics	Height 50 --------- Source of variation ---------				Height topping --------- Source of variation ---------			
		loc	rep(loc)	method	loc x method	loc	rep(loc)	method	loc x method
	DF	2	6	2	4	2	6	2	4
Line 1	MS	166.46	472.29	18.08	15.75	149.40	191.48	43.35	73.79
	F Value	3.74*	10.62***	0.41	0.35	2.25	2.88*	0.65	1.11
	DF	2	6	2	4	2	6	2	4
Line 2	MS	40.41	193.43	237.04	42.74	182.97	432.58	84.12	96.16
	F Value	0.87	4.15**	5.09*	0.92	4.61*	10.9***	2.12	2.42
	DF	2	6	2	4	2	6	2	4
Line 3	MS	93.07	359.77	154.04	25.57	374.26	332.80	80.37	24.31
	F Value	4.2*	16.25***	6.96**	1.15	11.38**	10.12***	2.44	0.74
	DF	2	6	2	4	2	6	2	4
Line 4	MS	23.01	353.32	78.74	12.35	397.798	318.1665	69.20182	11.57421
	F Value	0.52	7.96**	1.77	0.28	10.74	8.59	1.87	0.31
	DF	2	6	1	2	2	6	1	2
Line 5	MS	12.24	147.49	270.28	0.87	227.40	286.86	128.05	3.44
	F Value	0.31	3.75	6.87*	0.02	4.65	5.86*	2.62	0.07
	DF	2	6	1	2	2	6	1	2
Line 6	MS	75.02	82.45	31.63	145.65	203.02	92.48	81.58	67.39
	F Value	2.15	2.36	0.91	4.17	15.58**	7.1*	6.26*	5.17*
	DF	2	6	1	2	2	6	1	2
Line 7	MS	22.71	142.97	2.08	107.18	124.85	92.04	0.01	71.10
	F Value	0.96	6.04*	0.09	4.53	2.63	1.94	0.00	1.50
	DF	2	6	1	2	2	6	1	2
Line 8	MS	78.65	243.61	56.43	54.95	83.03	203.82	71.32	92.33
	F Value	11.81**	36.58***	8.47*	8.25*	2.10	5.15*	1.80	2.33

(*), (**), (***) - Differences between lines within the same triplet significant at P<0.05, 0.01, 0.0001, respectively.

Table 3.51 - Analysis of variance of differences between methods within the lines of the TN 97LC population, for the agronomic traits leaf length and leaf width.

TN 97LC Triplets	statistics	Leaf Length --- Source of variation --- Loc	rep(loc)	method	loc x method	Leaf Width --- Source of variation --- loc	rep(loc)	method	loc x method
	DF	2	6	2	4	2	6	2	4
Line 1	MS	521.01	11.19	1.53	13.93	117.80	3.58	9.81	6.47
	F Value	68.41***	1.47	0.20	1.83	40***	1.22	3.33	2.20
	DF	2	6	2	4	2	6	2	4
Line 2	MS	549.14	8.96	2.68	10.41	83.84	3.83	19.76	1.11
	F Value	78.81***	1.29	0.38	1.49	22.84***	1.04	5.38*	0.3
	DF	2	6	2	4	2	6	2	4
Line 3	MS	421.15	13.59	10.39	5.90	49.37	1.79	6.48	0.29
	F Value	84.38***	2.72*	2.08	1.18	15.85***	0.57	2.08	0.09
	DF	2	6	2	4	2	6	2	4
Line 4	MS	411.04	25.63	6.23	1.69	86.50	0.85	22.30	1.04
	F Value	58.68***	3.66*	0.89	0.24	29.5***	0.29	7.61**	0.35
	DF	2	6	1	2	2	6	1	2
Line 5	MS	256.56	9.60	28.13	0.48	37.17	4.10	4.35	3.28
	F Value	44.7**	1.67	4.90	0.08	30.45**	3.36	3.57	2.69
	DF	2	6	1	2	2	6	1	2
Line 6	MS	133.70	16.69	22.60	5.89	13.35	5.66	12.78	3.58
	F Value	31.23**	3.90	5.28	1.38	4.64	1.97	4.44	1.24
	DF	2	6	1	2	2	6	1	2
Line 7	MS	276.83	3.69	0.31	0.99	38.82	1.98	0.27	0.55
	F Value	19.17**	0.26	0.02	0.07	6.61*	0.34	0.05	0.09
	DF	2	6	1	2	2	6	1	2
Line 8	MS	237.43	13.50	18.00	4.46	52.01	5.19	2.98	1.08
	F Value	27.23**	1.55	2.06	0.51	22.89**	2.28	1.31	0.48

(*), (**), (***) - Differences between lines within the same triplte significant at P<0.05, 0.01, 0.0001, respectively.

Table 3.52 - Analysis of variance of differences between methods within the lines of the TN 97LC population, for the agronomic traits number of leaves per plant and yield.

TN 97LC Triplets	statistics	Leaf Number --------- Source of variation --------- loc	rep(loc)	method	loc x method	Yield ---------- Source of variation ---------- loc	rep(loc)	method	loc x method
	DF	2	6	2	4	2	6	2	4
Line 1	MS	19.41	11.36	1.19	3.11	802747.8	215926.6	18252.4	93455.1
	F Value	13.53**	7.92**	0.83	2.17	20.03***	5.39**	0.46	2.33
	DF	2	6	2	4	2	6	2	4
Line 2	MS	0.75	12.95	1.08	2.59	866474.0	60077.1	1207.1	151110.5
	F Value	0.31	5.37**	0.45	1.07	16.46***	1.14	0.02	2.87*
	DF	2	6	2	4	2	6	2	4
Line 3	MS	28.89	7.72	2.77	1.32	1647873.8	83016.1	109279.6	25218.0
	F Value	22.89***	6.12**	2.19	1.04	21.55***	1.09	1.43	0.33
	DF	2	6	2	4	2	6	2	4
Line 4	MS	10.17	3.00	5.36	0.78	789672.4	135556.7	15865.5	46828.2
	F Value	8.79**	2.59*	4.63*	0.67	20.26***	3.48*	0.41	1.2
	DF	2	6	1	2	2	6	1	2
Line 5	MS	9.78	7.84	2.23	0.16	1000575.7	47029.0	3749.5	49508.4
	F Value	6.39*	5.12*	1.45	0.11	47.92**	2.25	0.18	2.37
	DF	2	6	1	2	2	6	1	2
Line 6	MS	10.36	1.00	0.40	3.29	251072.6	104309.0	867.1	48487.2
	F Value	10.47*	1.01	0.40	3.32	10.38*	4.31*	0.04	2.00
	DF	2	6	1	2	2	6	1	2
Line 7	MS	2.85	5.24	0.01	1.19	396032.4	28138.9	418560.7	3937.0
	F Value	2.36	4.32*	0.00	0.99	5.51*	0.39	5.82	0.05
	DF	2	6	1	2	2	6	1	2
Line 8	MS	3.91	6.82	0.45	0.52	267618.2	147111.0	822.0	65877.7
	F Value	4.04	7.05*	0.46	0.54	2.33	1.28	0.01	0.57

(*), (**), (***) - Differences between lines within the same triplet significant at P<0.05, 0.01, 0.0001, respectively.

Table 3.53 - Agronomic trait means for lines of the TN 97LC family and separation of means within triplets based on Fischer's LSD.

TN 97LC triplet	Method	Height 50 (cm)		Height topping (cm)		Leaf length (cm)		Leaf width (cm)		Leaf number (No)		Yield (Kg/ha)	
	AD(F_1)	80.1	a	124.4	a	60.3	a	29.3	a	19.4	a	3035	a
1	Hybrid	80.3	a	127.3	a	59.5	a	27.4	b	20.1	a	3003	a
	MD(F_1)	78.2	a	123.5	a	59.7	a	28.9	a	19.6	a	3078	a
	AD(F_1)	74.6	b	117.6	a	60	a	28.8	a	19.6	a	2944	a
2	Hybrid	83.7	a	122	a	59.9	a	26.8	b	20.1	a	2953	a
	MD(F_1)	75.9	b	122.8	a	60.7	a	26.3	b	20.2	a	2964	a
	AD(F_1)	80.8	b	121.2	b	61.0	a	28.9	a	19.2	a	3139	a
3	Hybrid	85.7	a	126.7	a	60.5	a	27.3	b	20.1	a	3034	a
	MD(F_1)	78.5	b	122.4	ab	62.2	a	28.0	ab	19.1	a	2950	a
	AD(F_1)	78.6	a	124.1	a	60.7	a	29.6	a	20.5	ab	3101	a
4	Hybrid	82.9	a	125.3	a	59.3	a	27.2	b	21	a	3018	a
	MD(F_1)	77.8	a	120.9	a	60.7	a	29.8	a	19.7	b	3050	a
	AD(F_1)	76.6	b	117.7	a	62.9	a	28.1	a	19	a	2919	a
5	Hybrid	84.4	a	123.1	a	60.4	a	27.1	a	19.7	a	2948	a
	-	-	-	-	-	-	-	-	-	-	-	-	-
	AD(F_1)	79.6	a	119	b	61.8	a	28.6	a	19.5	a	2952	a
6	Hybrid	82.3	a	123.2	a	59.5	a	26.9	a	19.8	a	2938	a
	-	-	-	-	-	-	-	-	-	-	-	-	-
	AD(F_1)	80.7	a	122.0	a	60.5	a	27.1	a	19.7	a	3128	a
7	Hybrid	81.4	a	122.0	a	60.3	a	26.9	a	19.7	a	2823	a
	-	-	-	-	-	-	-	-	-	-	-	-	-
	AD(F_1)	80.6	b	119.2	a	61.8	a	27.8	a	19.9	a	3043	a
8	Hybrid	84.2	a	123.2	a	59.8	a	27.0	a	20.3	a	3029	a
	-	-	-	-	-	-	-	-	-	-	-	-	-

Means followed by the same letter within a column and within a KT 204LC line are not significantly different at the 5% level of significance.

leaves than the check. No significant differences were detected between any of the MD(F_1) lines and their respective TN 97LC checks for yield per hectare.

3.3.4 Relationships between DH parental lines and the DH-derived hybrids

In comparing the performance of the TN 90LC parental line families versus their performance when used as the pollinators for KT 204LC hybrids, most of the variability present in the inbred lines was eliminated or greatly reduced in the hybrids. The statistical analyses and mean values for the TN 90LC parental lines are given in Tables 3.8 and 3.9, respectively. The average performance of the ten TN 90LC ADDH lines was statistically significantly inferior to TN 90LC check for all variables except leaf length and leaf number. In comparison to TN 90LC, the average performance of the MDDH lines was significantly superior for plant height at the 50th day and after topping, but inferior for leaf width (tables 3.8 and 3.9). However, when used as the pollinators for AD(F_1) and MD(F_1) KT 204 hybrid populations, a statistically significant difference was noted only for leaf length in the AD(F_1) KT 204LC family (table 3.32). Although the AD(F_1) population was statistically superior to the KT 204LC check, the actual difference was only 0.82 cm, which is not visibly detectable.

There was also more variability between individual TN 90LC ADDH and MDDH lines parental lines compared to individual AD(F_1) and MD(F_1) KT 204LC lines. For the parental lines, among the ten ADDH lines statistically significant differences were observed for all traits except leaf number, and among the ten MDDH lines, statistically significant differences were observed for all traits (table 3.10). Conversely, when these parental lines were used as pollinators to form the KT 204LC hybrid, among the AD(F_1) KT 204LC hybrids differences were only statistically significant for plant height at the 50th day after transplanting and at topping (table 3.34). Among the MD(F_1) KT 204LC lines, a statistically significant difference was detected only for plant height after topping. Of particular interest is the performance of TN 90LC ADDH line five and MDDH line five as inbred lines, compared to how they did in hybrid combinations. Among 30 total parental lines evaluated, these two inbred

parental lines ranked 29th or 30th for all agronomic traits measured (tables 3.11 through 3.13). Remarkably however, both lines performed well in KT 204LC hybrid combinations, particularly with regard to yield per hectare (tables 3.47 through 3.49). Among the 30 total hybrid lines evaluated, AD(F_1) and MD(F_1) KT 204LC line five ranked 7th and 30th for plant height at the 50th day after transplanting, 5th and 30th for plant height after topping, 30th and 4th for leaf length, 30th and 26th for leaf length 1st and 28th for leaves per plant, and 15th and 6th for yield, respectively.

In comparing the performance of the GR 149LC ADDH and MDDH parental line families versus their GR 149LC inbred source, statistically significant differences were observed for most of the agronomic traits measured (table 3.20). The mean performance of the ADDH lines was statistically different from GR 149LC for all traits except leaf length and yield. For the traits that were statistically significant, the ADDH lines were inferior for all traits except leaf length, which was greater than for the GR 149LC source (table 3.21). The mean performance of the GR 149LC MDDH lines was statistically inferior to GR 149LC for plant height at 50 days after transplanting, leaf width, and leaf number; no significant differences were observed for the other three traits (tables 3.20 and 3.21).

Unlike what was seen for the TN 90LC and KT 204LC families, when the GR 149LC ADDH and MDDH lines were used as the pollinators for AD(F_1) and MD(F_1) TN 97LC hybrid populations, there were substantial differences between the hybrid populations in comparison to the TN 97LC check. For the eight TN 97LC AD(F_1) hybrids, statistically significant differences from the check were detected for all traits except yield (table 3.44). Similarly, the average performance of the four MD(F_1) hybrids was statistically different from TN 97LC for all traits except leaf length and yield (table 3.44). However, these results are somewhat misleading since the AD(F_1) and MD(F_1) TN 97LC hybrids were inferior to the TN 97LC check only for plant height and leaf number. Both the AD(F_1) and the MD(F_1) hybrids were actually superior to TN 97LC for leaf length and leaf width (table 3.45). As a result, the average yield was not significantly different among the three TN 97LC populations.

As was seen for the TN 90LC parental lines versus KT 204LC hybrid line comparisons, there was also more variability between individual GR

149LC ADDH and MDDH parental lines compared to individual AD(F_1) and MD(F_1) TN 97LC hybrid lines. Among the eight ADDH GR 149LC lines, statistically significant differences were detected for leaf length, leaf width, and leaf number; among the four GR 149LC MDDH lines, statistically significant differences were detected for all traits except plant height after topping (table 3.22). However, when the GR 149LC ADDH and MDDH lines were used as pollinators to make hybrid lines, the only statistically significant difference among the TN 97LC AD(F_1) or MD(F_1) hybrids was for leaf width among the four MD(F_1) hybrid lines (table 3.48).

3.4 Conclusions

The anther culture (ADH method) was considerably more efficient than the interspecific hybridization (MDH technique) to generate haploid plants in burley tobacco. The response of the ADH method, in terms of number of haploids produced was superior to the response of the MDH method. In addition to the overall low efficiency, the MDH method displayed considerable differential response between TN 90LC and the GR 149LC genotypes.

The ADDH TN 90LC population was inferior to the inbred TN 90LC for several agronomic traits, including yield of cured leaves per hectare. The inferiority of the ADDH TN 90LC was not observed in the ADDH GR 149LC populations, and for both genotypes, the MDDH populations were comparable to the inbred parental genotypes. In comparing the MDDH populations versus the inbred source for the TN 90LC and the GR 149LC background, both MDDH populations were taller than the inbred checks, but the difference was statistically significant only for TN 90LC. Nielsen and Collins, (1989) observed that three out of four five-lines sets of KY 17 MDDH were also not significantly different from inbred source lines; one set was actually statistically inferior to the inbred population. As observed in the TN 90LC experiment, the plant height after topping of the MDDH populations were significantly superior compared to the inbred sources. The ADDH populations of both genotypes were inferior, but only the TN 90LC ADDH population was significantly inferior.

There are numerous reports in the literature about the inferiority of the ADDH method versus the MDDH technique for producing homozygous lines compared to their inbred sources. The results obtained in the TN 90LC experiment corroborate those findings, but the trend was not observed in the GR 149LC trials. Studies using several genotypes and the respective DH lines showed that there are situations where the ADDH population is comparable or even superior to the inbred sources. In a test with the flue-cured hybrid “DH 10”, 47 ADDH lines were compared to the parental hybrid. Three of those ADDH lines were superior to the hybrid DH 10, but the overall performance was unsatisfactory (Smalcelj and Perica, 2000). In a study with three different oriental tobacco cultivars in Southeast Europe, two of the diploid sources were superior to the average yield of their ADDH lines. In one of the genotypes, the ADDH lines were superior to the source cultivar, in 14.2 % (Miceska, 2009).

The relatively poor yielding ability of ADDH lines reported in the literature is consistent with the findings of this research for TN 90LC ADDH populations. Differential response was observed from different genotypes, within the same type of tobacco. There was considerable variability on how the DH lines derived from a specific plant (and even from a specific haploid source) perform. The present study confirmed the overall inferiority of the DH method for TN 90LC, but several individual TN 90LC ADDH lines were equal or superior to the inbred TN 90LC source.

The relative performance of hybrid lines derived from DH sources was better in comparison to the hybrid checks than the relative performance of DH parental lines in comparison to the inbred checks. The relative performance of the AD(F_1) KT 204LC hybrids in comparison to the KT 204LC check was superior to the relative performance of the ADDH TN 90LC parental lines in comparison to the TN 90LC source for most agronomic traits, especially for yield. For the GR 149LC and TN 97LC populations, no differences for yield were seen among the three GR 149LC parental line populations, or among the three TN 97LC hybrid line populations. For the remaining agronomic traits, there were considerably more statistical differences noted between methods and among individual lines for the parental lines than were observed for the hybrid lines. The variation observed between the ADDH and MDDH

lines (within the same population) were larger than the differences observed between the DH-derived hybrids. Overall, the AD(F_1) and MD(F_1) lines were superior to their parental DH lines, compared to their respective checks, and were also more uniform.

Previous research studies suggested that the inferiority of DH lines could be due to two main factors: loss of residual heterozygosity or genetic mutations induced by the tissue culture methodology. Several investigators have explained the differences between doubled-haploid lines and their source cultivars by a loss of residual heterozygosity in the inbred cultivars (Collins et al. 1973; De Paepe et al. 1977). Residual heterozygosity can also explain the differences observed in this study. According to this theory, only the inbred cultivars with heterozygous loci for a particular character could produce an anther-derived line that was significantly different for that character (a genotypic effect). Also, those cultivars could fix a gene combination in their doubled haploids for performance above or below the cultivar performance. In a study by Deaton et.al, 1982, significant differences between doubled haploid lines and their parental cultivars were found only for certain genotypes and characters. In addition, the doubled haploid lines were distributed in both directions around the performances of their source cultivars for yield, days to flower, and leaf number. The hypothesis that the variation is fixed at the haploid production step is consistent with the proposal that reduced vigor is due to variants that preexist in the gametic pool instead of changes induced in the culturing system. Deaton et.al. (1986) suggested that the majority of variation observed in anther-derived materials is a result of differences that were stabilized when the haploids were cultured to produce doubled haploids.

However, some early studies suggest that the inferiority of DH lines might be associated with mutations that may have occurred due to the use of colchicine in the chromosome doubling process. However, the inferiority of DH lines has also been reported in studies where tissue culture methods were used to double the chromosome number without the use of colchicine, similar to the methods used in the present research. Deaton et al. (1982); Arcia & Wernsman, (1978) suggested that the genetic variation affecting DH lines

may arise prior to chromosome doubling, as a result of intrinsic mutagenic effects of the tissue culture process.

Results from the present study suggest that mutagenic events may occur in either the anther culture process or the chromosome doubling process, or possibly both. In the present study, within the TN 90LC DH populations one ADDH (line 5) and one MDDH (line 5) line were both grossly inferior to the TN 90LC check. The magnitude of agronomic inferiority for both of these particular ADDH and MDDH lines suggests that at least one mutation event may have taken place at some point during the DH process. If the DH process was at some point mutagenic, and the resulting mutant genes were inferior to normal alleles in the conventional pure line parent, the DH lines would be expected to be inferior to selfed progenies of the conventional source parental lines. In the present study, if mutations were responsible for the poor agronomic performance of the TN 90 ADDH5 and MDDH5 lines, the mutations must have been recessive since both inferior parental lines produced acceptable KT 204LC hybrid lines.

In the ADDH method, plants go through four tissue culture steps (induction of haploids, rooting of haploids, DH induction from haploid tissues and rooting of DH plantlets). There are only two tissue culture steps for the MDDH technique (DH induction from haploid tissues and rooting of DH plantlets). As suggested by Deaton *et. al.* (1982), mutations could be a source of variation occurring before chromosome doubling. The two extra tissue culture steps to which the ADDH plants are subjected could have an impact in increasing mutations, which may have a negative effect on overall ADDH performance and explain why, on average, ADDH derived lines are inferior to MDDH lines. However, the present study indicates that differences between the two methods are minimal when the DH lines from either method are used as parental lines to generate hybrid varieties. The improved relative performance of both ADDH and MDDH lines in hybrid combinations versus their performance as inbred lines suggests that the generally inferior performance of DH lines may be due to recessive mutations that may occur in the anther culture and/or doubled haploid tissue culture process(es).

Appendix Chapter 3

List of Tables

Table A 3.1 – Analysis of variance of the interactions between subplot variables (split plot design) for six agronomic traits, among all methods included in the TN 90LC family.

Interaction	Statistics	Height 50	Height Topping	Leaf length	Leaf width	Leaf Number	Yield
line* method	DF	16	16	16	16	16	16
	MS	163.64	68.4	7.34	3.28	0.91	211984
	F value	2.07**	1.06	1.04	1.41	0.9	2.10*
Location* method	DF	4	4	4	4	4	4
	MS	176.32	102.99	19.64	3.36	0.85	178793
	F value	2.23	1.59	2.77*	1.44	0.83	1.77
Location* line* method	DF	32	32	32	32	32	32
	MS	71.37	30.61	3.93	2.32	0.56	117285
	F value	0.9	0.47	0.56	1	0.55	1.16

(*), (**), (***) - Differences significant at P<0.05, 0.01, 0.0001, respectively

Table A 3.2 – Analysis of variance of the interactions between subplot variables (split plot design) for six agronomic traits. Differences between the ADDH and the inbred populations of TN 90LC family.

Interaction	Statistics	Height 50	Height Topping	Leaf length	Leaf width	Leaf Number	Yield
line* method	DF	8	8	8	8	8	8
	MS	78.79	56.67	6.57	3.6	0.82	248277
	F value	1.13	0.79	1.02	1.84	0.65	2.18*
Location* method	DF	2	2	2	2	2	2
	MS	96.33	169	17.37	3.35	1.17	146220
	F value	1.38	2.35	2.7	1.71	0.93	1.29
Location* line* method	DF	16	16	16	16	16	16
	MS	43.37	22.94	4.34	2.27	0.27	134224
	F value	0.62	0.32	0.68	1.16	0.22	1.18

(*), (**), (***) - Differences significant at P<0.05, 0.01, 0.0001, respectively

CHAPTER 4: Optimal Generation to Develop Tobacco Doubled Haploids When Selecting For Quantitatively Inherited Traits

4.1. Introduction

Choosing the best generation in which the production of doubled haploids (DH) should be initiated is a paradigm when considering quantitatively inherited traits in plants. The most efficient pathway is to produce haploids as early as possible, i.e. from the F_1 generation. Conversely, the most efficacious manner is to delay the haploid induction process for several generations, allowing more recombination events and permitting phenotypic selection. In a simulation study with maize, Bernardo (2009) showed that F_2-derived DH lines sustained higher long-term genetic gains compared to DH lines derived from the F_1 generation; this was attributed to selection practiced in the segregating F_2 population before the induction of haploids.

Li et al. (2013) evaluated genetic gains for a yield and adaptation trait (combined index of various traits, such as maturity, plant height, yield components, and quality, which can be selected for in early generations before yield trials begin) in wheat, comparing a selected bulk method (SELBLK) with F_1 and F_3-derived DH lines. In general, the F_1-derived DH lines showed higher genetic gains for yield but lower gains for the adaptation trait, which could be explained by the lower selection intensity applied to yield and the time efficiency of the DH strategy. The performance of DH lines in this study was different from Bernardo (2009), because the former study considered the possibility of advancing two generations per year, resulting in poor economic and genetic efficiency of the DH strategy compared to SELBLK.

Charmet and Branlard (1985) reported no differences in most of the yield components in triticale (*X triticosecale*) between DH lines derived from F_1 plants and selfed lines inbred through the single seed descent method. The findings indicate that similar ranges of recombination over several generations of selfing can be achieved in F_1-derived DH, which suggests that there is no need to delay the induction of haploids until the F_2 generation. Jannink and

Abadie (1999) showed that the DH method provided the biggest short-term genetic gain, but it was inferior to the single seed descent method in the long-term. The complexity in determining the genetic and economic efficiency of most breeding methods are based on the interaction of a considerable number of factors, such as plant species, number of generations per year, number of alleles controlling the trait, linkage, selection intensity and the frequency of alleles.

Quantitatively Inherited Disease Resistance

Oomycetes, fungi and bacteria are the common microorganisms responsible for soil-borne diseases in tobacco. Breeding resistant varieties is one of the most effective control measures to minimize those losses. Frequently, the mechanisms of resistance to certain diseases and races is quantitatively inherited; i.e. controlled by several genes. The influence of multiple genes over one single trait increases the complexity in achieving highly resistant varieties, compared to traits controlled by a single gene. The quantitatively inherited traits considered in this study were resistance to black shank and *Fusarium* wilt.

Black shank is caused by the oomycete *Phytophthora nicotianae* (van Breda de Hann), which primarily infects roots, but also affects stalks and leaves of all types of tobacco causing stunting and plant death at any stage of development (Shew et al., 1991; Gallup et al., 2006). It is the most important burley and dark tobacco disease in the USA, causing considerable losses every year (Pearce et al., 2013). Three races of *P. nicotianae* were identified in the USA (Apple, 1962) where the most important are the race 0 and race 1.

Race 0 is considered the wild type and occurs in all tobacco-growing regions, being the most virulent for burley, and predominant in flue-cured tobacco areas in NC since 1931(Shew et al., 1991). Race 1 was reported in the USA in 1954 in breeding lines of burley tobacco in Kentucky. The increase in predominance of this race has followed the race shift forced by the use of cultivars containing *Php* and *Phl* genes, which are single gene mechanisms conferring complete resistance to race 0 but no resistance to race 1 (Sullivan et al., 2005). *P. nicotianae* race 3 was reported in the state of Connecticut in 2010 in a cigar-wrapper tobacco (Gallup and Shew, 2010). Race 2 has been

described only in South Africa, but is not considered epidemic (Prinsloo and Pauer, 1974).

Another quantitatively inherited soil-borne disease which affects a broad range of plant species worldwide is *Fusarium* wilt. It usually infects scattered tobacco plants in the field and may occur in all types of soils, but the incidence is often associated with wet areas and sandy soils, such as river bottoms. In the USA, *Fusarium* wilt was first reported in the state of Maryland in 1921, but it is now widely dispersed in all tobacco areas (Shoemaker and Shew, 1999). The disease is caused by the ascomycete *Fusarium oxysporum f. nicotianae* (J. Johnson) W.C. Snyder & H.N. Hansen 1971(anamorphic Gibberella). This is an example of a soil inhabitant, with capacity to survive more than 10 years in the soil as chlamidospores. When environmental conditions are optimal and actively growing tobacco roots are present, the chlamidospores are able to germinate because nutrients released from tobacco plants into the rhizosphere create a suitable environment for fungus germination, growth and multiplication (Shew and Lucas, 1991). Other factors such as temperature, soil moisture and activity of tobacco cyst nematode and root-knot nematode may influence the severity of the disease.

DH lines are usually generated from greenhouse grown plants in the F_1 generation in order to minimize the time required to reach homozygosity. The haploid or DH plants are then screened for resistance; although the best DH lines can subsequently be identified in disease nurseries, true selection is not possible because disease resistance is fixed due to the homozygosity of the DH lines. When quantitatively inherited traits for soil-borne diseases are considered, it may be better to delay the DH process until the F_2 generation. Segregating F_2 plants can be grown in field nurseries having high levels of the soil-borne disease of interest, with only those plants displaying high disease resistance being selected for use in the DH process. In essence, the plants from the segregating F_2 line are “prescreened” for quantitatively inherited disease resistance.

4.2. Materials and Methods

4.2.1 Population Development

Three hybrid lines were developed for this study; to facilitate descriptions and citations, the lines will be referred to as EZ1, EZ2 and EZ3. Inbred line TKF 7002H was used as a common female parent in each of the three hybrid crosses. This female line is characterized as having high resistance to race 0 and race 1 black shank, *Fusarium* wilt, and bacterial wilt (*Ralstonia solanacearum* Smith), but it is susceptible to potato virus Y (PVY – Potyvirus group), tobacco mosaic virus (TMV – Tobamovirus group), blue mold (*Peronospora tabacina* Adam), and black root rot [*Thielaviopsis basicola* (Berk. and Broome) Ferraris]. In addition, TKF 7002H does not carry the single gene dominant trait that infers immunity to race 0 black shank.

The paternal lines TKF 4028A12 and TKFE 4028C5 used in hybrids EZ1 and EZ2, respectively, are resistant to blue mold, PVY, TMV, black root rot, wildfire (*Pseudomonas tabaci* Stevens); however, they have only low to moderate resistance to both races of black shank. The third paternal line, TKF 2002B6 used for EZ3, is known to be immune to race 0 and highly resistant to race 1 black shank, wildfire, PVY, and black root rot. As mentioned earlier, the target of each cross was the development of DH lines having unique combinations of black shank and *Fusarium* wilt resistance with resistance to the other diseases provided by each individual paternal line. Based on the characteristics of the parental lines, the relative resistance expected for EZ1, EZ2 and EZ3 populations for several diseases is presented in Table 4.1.

All crosses to produce EZ1, EZ2 and EZ3 hybrid lines were made on August 31, 2011 at Spindletop Research Farm near Lexington, KY. The F_1 seed of the three hybrid lines were harvested on September 30, 2011. A portion of the seed of each hybrid was seeded in the greenhouse on October 11, 2011 to generate the F_2 populations. Twenty-eight days after seeding, 128 F_1 plants of each hybrid were transplanted into 128-cell trays of expanded polystyrene and placed on a hydroponic solution containing 100ppm 5-10-15 fertilizer. Terramaster® 4EC was also added to the solution to speed up flowering and production of F_2 seed. Etridiazole is the active ingredient of the

fungicide Terramaster® 4EC, which is recommended for the prevention or cure of *Pythium* root rot in tobacco seedbeds. Etridiazole belongs to the group of triazols, which are known for their inhibitory effect on plant growth when used in higher than recommended concentrations. An overdose of etridiazole inhibits biosynthesis of the hormone gibberellin, which is responsible primarily for shoot and stem elongation, resulting in early flowering of tobacco plants. In the current study, Terramaster® 4EC was used at 20 times the recommended concentration in the EZ1-F_1, EZ2-F_1 and EZ3-F_1 hydroponic solution. This concentration induced premature flowering, enabling the harvest of F_2 seed capsules as early as 110 days after seeding, or 82 days after the Terramaster® was added to the hydroponic solution.

4.2.2 Doubled Haploid Materials

Two experiments were conducted to compare the effectiveness of utilizing the F_1 versus F_2 generation for the development of DH lines having high quantitatively inherited resistance to soil-borne diseases. The first experiment investigated resistance to race 1 black shank, while the second experiment investigated *Fusarium* wilt resistance. For both experiments, thirty plants from each of the three EZ hybrid crosses were grown in the field without pre-selection for disease resistance; these plants were used to produce haploid lines from the F_1 generation. Using protocols described in Chapter 3, anthers were selected from these thirty F_1 hybrid plants in August 2012 to produce ADH plants; the plants were also pollinated with *N. africana* to produce MDH plants. From the F_1 generation, the total number of haploid plants produced was: ten ADH and ten MDH plants from cross EZ1; ten ADH and eight MDH plants from cross EZ2; and ten ADH and ten MDH plants from cross EZ3.

The development of ADDH and MDDH doubled haploids was carried out in a laboratory using the protocol described in Chapter 3 (sections 3.2.1.1, 3.2.1.2 and 3.2.1.3). The development of haploid plants and DH populations from both methods were carried out in the tissue culture laboratory and in the greenhouses from August 2012 to February 2014, to allow the collection of seed for field studies.

Table 4.1 - Expected levels of disease resistance of each one of the hybrid lines

Disease	Hybrid lines		
	EZ1	EZ2	EZ3
Black Shank Race 0*	low / medium	low / medium	high
Black Shank race 1*	Low / medium	low / medium	medium / high
Fusarium Wilt *	low	low	medium / high
Bacterial Wilt *	low	low	medium / high
Black Root Rot	susceptible	susceptible	high
PVY	high	high	high
Blue Mold	high	high	susceptible
TMV	high	high	susceptible

* Denotes quantitatively inherited disease resistance

For the black shank resistance experiment, the production of haploid plants from the F_2 generation was carried out in a race 1 black shank nursery. The segregating EZ1 - F_2 and EZ2 - F_2 lines, derived from F_1 progeny seed described in the section 4.2.1, were transplanted to a nursery near Greeneville, TN, that had a history of very high levels of race 1 black shank. One hundred and twenty plants from both the EZ1 - F_2 and EZ2 - F_2 populations were transplanted in the nursery on June 4, 2012. Flower buds from four highly resistant EZ1 - F_2 plants and three highly resistant EZ2 - F_2 plants were harvested in August 2012 to produce ADH plants. In the same time period, ten plants from each EZ hybrid line that exhibited high resistance to black shank (including the ones used for ADH) were selected and crossed with *N. africana* to allow the production of MDH plants.

For the *Fusarium* wilt experiment, the EZ2 - F_2 and EZ3 - F_2 lines were transplanted in a nursery near Owensboro, KY, that had a history of moderate *Fusarium* wilt pressure. Sixty plants of segregating EZ2 - F_2 and 75 plants of EZ3 - F_2 were transplanted to the nursery on June 13, 2012. From both the EZ2 - F_2 population and the EZ3 - F_2 population, six plants that displayed high resistance to *Fusarium* wilt were selected and used as the source of anthers for the production of ADH plants. For the production of MDH plants, ten plants that displayed high resistance to *Fusarium* wilt (including the six plants used for ADH production) were selected from both the EZ2 - F_2 population and the EZ3 - F_2 population and used as female parents for interspecific crosses with *N. africana*. The harvest of flower buds and interspecific crosses were made in late August and early September, 2012.

From the F_2 generation, for the black shank study the total number of haploid plants produced was: ten ADH and five MDH plants from cross EZ1; and ten ADH and seven MDH plants from cross EZ2. For the *Fusarium* wilt study, the total number of haploid plants produced from the F_2 generation was: eight ADH and seven MDH plants from cross EZ2; and ten ADH and six MDH plants from cross EZ3. The production of ADDH and MDDH lines from these ADH and MDH plants was carried out in a laboratory using the protocol described in Chapter 3.

4.2.3 Field Trials

During the 2014 growing season, field trials were carried out to evaluate the performance of the DH lines derived from F_1 versus F_2 generations. The F_2 derived DH lines selected for race 1 black shank resistance and theirs respective DH lines derived from the F_1 generation were evaluated in three different race 1 black shank nurseries. All ten ADDH lines from each EZ1 - F_1, EZ1 - F_2, EZ2 - F_1 and EZ2 - F_2 generation were transplanted in the Birdwell nursery (BW) near Greeneville, TN; this was the same nursery used to select the race 1 black shank F_2 population of both EZ1 and EZ2 lines. The MDDH lines of EZ1 - F_1, EZ1 - F_2, EZ2 - F_1 and EZ2 - F_2 were tested in the Hunter nursery (HT) near Greeneville, TN. The third location in which both generations of all ADDH and all MDDH of EZ1 and EZ2 lines were evaluated for resistance to race 1 black shank was in the Franklin County, KY, nursery (FC). The plots were transplanted as a randomized complete block design with three replications in BW, on May 29 2014 and HT on May 30 2014. Four replications were used in FC, which was transplanted on June 04 2014. Plots consisted of 20 plants in BW and HT and 18 plants in FC.

Fusarium wilt evaluations consisted of only one nursery near Owensboro, KY (OW), which was transplanted as a randomized complete block with 25 plants per plot. Due to space limitations, only lines of the ADH method were tested in this nursery. The ten lines of ADDH EZ2 - F_1, eight of EZ2 - F_2, ten of EZ3 - F_1 and ten of EZ3 - F_2 were transplanted on June 09 2014.

Initial stand counts were recorded for each plot in all nurseries two weeks after transplanting. Disease incidence (% symptomatic plants) was estimated by recording the number of symptomatic plants at the 4^{th}, 6^{th}, 8^{th}, 10^{th} and 12^{th} week after transplanting (WAT).

4.2.4 Data Analysis

Because the initial number of plants per plot varied across plots and locations, statistical analyses were performed based on the percentage of

symptomatic plants and not on the absolute number of plants per plot. Analysis of variance (ANOVA) was performed on disease incidence data (percentage of symptomatic plants) using the PROC GLM procedure of SAS (Version 9.3, SAS Institute, Cary, NC).

For black shank incidence, combined data from all locations were analyzed as repeated measurements using the following model for all five times data were recorded (4 WAT, 6 WAT, 8 WAT, 10 WAT, 12 WAT):

$$Y_{ikl} = \mu + L_i + G_k + N_l + NG_{k(l)} + LG_{ik} + LNG_{ik(l)} + E_{ikl}$$

Where:

Y_{ikl} = the observation of the k^{th} genotype and l^{th} generation at the i^{th} location,

μ = overall mean,

L_i = the effect of the i^{th} location,

G_k = the effect of the k^{th} genotype,

N_l = the effect of l^{th} generation,

$NG_{k(l)}$ = the effect of k^{th} genotype nested within l^{th} generation;

LG_{ik} = the effect of the interaction of i^{th} location and k^{th} genotype,

$LNG_{ik(l)}$ = the effect of the interaction of i^{th} location and k^{th} genotype nested within l^{th} generation,

E_{ikl} = the residual error

For the data analysis of the incidence of black shank at individual locations and for incidence of *Fusarium* wilt, the ANOVA was performed as repeated measurements using the following model for all five times data were recorded (4 WAT, 6 WAT, 8 WAT, 10 WAT, 12 WAT):

$$Y_{kl} = \mu + G_k + N_l + NG_{k(l)} + E_{ikl}$$

Where:

Y_{kl} = the observation of the k^{th} genotype and l^{th} generation,

μ = overall mean,

G_k = the effect of the k^{th} genotype,

N_l = the effect of l^{th} generation,

NG_{kl} = the effect of the interaction of k^{th} genotype and l^{th} generation;

E_{ikl} = the residual error

4.3 Results and Discussion

4.3.1 Disease Incidence in the DH Populations - Location effects

Black Shank

The percentage of plants infected by black shank in populations of the EZ1 genotype was equal or higher for the F_1 generation compared to the F_2 generation, for all five weeks measured. For the EZ1 F_1 and F_2 generation this trend was observed. For the ADDH population at the BW location (table 4.2), the MDDH at the HT location (table 4.3), the ADDH at FC (table 4.4) and the MDDH at the FC location (table 4.5).

BW was the location where plants had the highest disease incidence (table 4.2), followed by HT (table 4.3). Both the ADDH and MDDH populations grown at the FC location were the least affected by black shank (table 4.4 and 4.5). It was known that the disease pressure was higher at the BW location and lower at FC, which was confirmed by the percentage of affected plants in the parental lines and in the DH populations.

The effect of location for the DH populations of the EZ2 family and its parental lines were very similar to the effects observed for the EZ1 genotype. The BW location had the highest incidence of black shank for both F_1 and F_2 generations of the EZ2 (table 4.6), and the FC location had the lowest incidence for both the ADDH and the MDDH populations (tables 4.8 and 4.9). The F_1 generation was more affected by black shank than the F_2 populations at the 12^{th} WAT at all locations.

With the exception of the F_1 generation at the BW location, all other DH populations (F_1 and F_2) displayed less than 5% of plants with black shank symptoms up to the 8^{th} WAT (tables 4.6, 4.7, 4.8 and 4.9). The EZ2 male parent, TKFE 4028C5, and the F_1 DH population were the most susceptible populations.

Table 4.2 – Incidence of black shank in ADDH lines of different generations of the EZ1 genotype, in five different weeks after transplant. BW Location, 2014.

Line	Weeks After Transplant				
	4th (%)	6th (%)	8th (%)	10th (%)	12th (%)
F_1 Generation DH lines					
EZ 51	0.0	2.7	10.6	52.6	89.5
EZ 52	0.0	0.0	0.0	0.0	2.7
EZ 53	0.0	0.0	2.7	8.2	62.4
EZ 54	0.0	1.8	3.5	5.3	55.4
EZ 55	0.0	0.0	3.5	3.5	56.8
EZ 56	0.0	1.8	1.8	7.0	38.6
EZ 57	0.0	1.9	1.9	3.5	14.1
EZ 58	0.0	1.8	1.8	7.0	52.4
EZ 59	0.0	0.0	0.0	1.8	30.5
EZ 60	0.0	0.0	0.0	3.4	40.0
MEAN	**0.0**	**1.0**	**2.6**	**9.2**	**44.2**
F_2 Generation DH lines					
EZ 92	0.0	0.0	0.0	0.0	10.6
EZ 93	0.0	0.0	0.0	2.7	15.8
EZ 94	0.0	0.0	0.0	0.0	23.9
EZ 95	0.0	0.0	0.0	5.3	62.3
EZ 96	0.0	0.0	0.0	0.0	0.0
EZ 97	0.0	0.0	0.0	0.0	5.3
EZ 98	0.0	0.0	0.0	0.0	5.3
EZ 99	0.0	0.0	1.9	1.9	32.6
EZ 100	0.0	0.0	0.0	0.0	15.8
EZ 101	0.0	1.7	5.1	13.5	68.9
MEAN	**0.0**	**0.2**	**0.7**	**2.3**	**24.0**
EZ1 parental lines					
Female	0.0	0.0	3.4	3.4	12.1
Male	0.0	2.8	8.7	24.3	60.4

Table 4.3 – Incidence of black shank in MDDH lines of different generations of the EZ1 genotype, in five different weeks after transplant. HT Location, 2014.

Line	Weeks After Transplant				
	4th (%)	6th (%)	8th (%)	10th (%)	12th (%)
F_1 Generation DH lines					
EZ 153	0.0	1.8	3.5	50.1	72.3
EZ 154	0.0	1.8	3.3	35.6	54.4
EZ 155	0.0	0.0	0.0	3.5	10.8
EZ 156	0.0	0.0	0.0	35.1	62.8
EZ 157	0.0	0.0	0.0	0.0	17.7
EZ 158	0.0	0.0	0.0	7.0	23.7
EZ 159	0.0	0.0	0.0	9.0	32.2
EZ 160	0.0	0.0	0.0	0.0	1.8
EZ 161	0.0	0.0	3.2	8.0	29.5
EZ 162	0.0	0.0	0.0	0.0	1.8
MEAN	**0.0**	**0.4**	**1.0**	**14.8**	**30.7**
F_2 Generation DH lines					
EZ 191	0.0	0.0	0.0	3.6	8.9
EZ 192	0.0	0.0	0.0	5.6	3.7
EZ 193	0.0	0.0	0.0	0.0	0.0
EZ 194	0.0	0.0	0.0	0.0	0.0
EZ 195	0.0	0.0	1.8	1.8	4.8
MEAN	**0.0**	**0.0**	**0.4**	**2.2**	**3.5**
EZ1 Parental lines					
Female	0.0	0.0	0.0	0.0	2.4
Male	0.0	0.0	0.0	29.8	56.8

Table 4.4 – Incidence of black shank in ADDH lines of different generations of the EZ1 genotype, in five different weeks after transplant. FC Location, 2014.

Line	Weeks After Transplant				
	4th (%)	6th (%)	8th (%)	10th (%)	12th (%)
F_1 Generation DH lines					
EZ 51	0.0	2.9	8.6	14.3	24.2
EZ 52	0.0	0.0	0.0	0.0	2.8
EZ 53	0.0	0.0	0.0	4.3	11.4
EZ 54	0.0	2.9	12.8	15.7	21.3
EZ 55	0.0	1.6	3.0	5.8	11.9
EZ 56	0.0	0.0	0.0	3.6	6.4
EZ 57	0.0	0.0	1.5	1.5	1.5
EZ 58	0.0	0.0	4.4	10.2	16.0
EZ 59	0.0	1.6	1.6	3.1	3.1
EZ 60	0.0	2.8	8.9	11.6	17.7
MEAN	**0.0**	**1.2**	**4.1**	**7.0**	**11.6**
F_2 Generation DH lines					
EZ 92	0.0	0.0	0.0	0.0	0.0
EZ 93	0.0	0.0	1.5	1.5	4.2
EZ 94	0.0	0.0	0.0	1.7	3.3
EZ 95	0.0	0.0	3.0	7.4	11.8
EZ 96	0.0	0.0	0.0	0.0	0.0
EZ 97	0.0	0.0	1.4	2.8	7.1
EZ 98	0.0	0.0	0.0	0.0	0.0
EZ 99	0.0	0.0	0.0	0.0	3.5
EZ 100	0.0	0.0	0.0	0.0	0.0
EZ 101	0.0	0.0	0.0	1.4	10.0
MEAN	**0.0**	**0.0**	**0.6**	**1.5**	**4.0**
EZ1 Parental Lines					
Female	0.0	1.5	1.5	2.9	2.9
Male	0.0	0.0	2.9	10.3	16.0

Table 4.5 – Incidence of black shank in MDDH lines of different generations of the EZ1 genotype, in five different weeks after transplant. FC Location, 2014.

Line	Weeks After Transplant				
	4th (%)	6th (%)	8th (%)	10th (%)	12th (%)
F_1 Generation DH lines					
EZ 153	0.0	1.4	7.0	34.3	40.0
EZ 154	0.0	0.0	0.0	11.8	25.0
EZ 155	0.0	0.0	5.0	6.4	13.7
EZ 156	0.0	0.0	1.8	14.2	17.1
EZ 157	0.0	0.0	1.6	0.0	4.4
EZ 158	0.0	0.0	0.0	5.7	14.3
EZ 159	0.0	3.1	3.1	6.3	21.6
EZ 160	0.0	0.0	1.6	3.3	3.3
EZ 161	0.0	0.0	0.0	0.0	4.2
EZ 162	0.0	0.0	0.0	1.8	3.6
MEAN	**0.0**	**0.5**	**2.0**	**8.4**	**14.7**
F_2 Generation DH lines					
EZ 191	0.0	0.0	0.0	0.0	4.7
EZ 192	0.0	0.0	0.0	1.4	1.4
EZ 193	0.0	0.0	0.0	0.0	3.0
EZ 194	0.0	0.0	0.0	0.0	0.0
EZ 195	0.0	0.0	0.0	0.0	1.4
MEAN	**0.0**	**0.0**	**0.0**	**0.3**	**2.1**
EZ1 Parental Lines					
Female	0.0	1.5	1.5	2.9	2.9
Male	0.0	0.0	2.9	10.3	16.0

Table 4.6 – Incidence of black shank in ADDH lines of different generations of the EZ2 genotype, in five different weeks after transplant. BW Location, 2014.

Line	Weeks After Transplant				
	4th (%)	6th (%)	8th (%)	10th (%)	12th (%)
F_1 Generation DH lines					
EZ 62	0.0	1.8	1.8	17.8	84.1
EZ 63	0.0	5.2	8.4	26.9	93.2
EZ 64	0.0	0.0	7	32.7	100
EZ 65	1.7	10.7	24.6	42.1	93
EZ 66	0.0	1.9	8.7	48.4	96.6
EZ 67	0.0	0.0	1.8	8.1	40.3
EZ 68	0.0	0.0	0.0	6.7	76.2
EZ 69	0.0	0.0	0.0	7	59.6
EZ 70	0.0	1.3	7	19.4	94.5
EZ 71	0.0	0.0	1.7	1.7	12.3
MEAN	**0.2**	**2.1**	**6.1**	**21.1**	**75**
F_2 Generation DH lines					
EZ 102	0.0	0.0	1.8	5.3	49.8
EZ 103	0.0	0.0	0.0	0.0	12.3
EZ 104	0.0	0.0	6.8	13.5	62.5
EZ 105	0.0	0.0	0.0	1.7	29.1
EZ 106	0.0	0.0	3.5	1.8	19
EZ 107	0.0	0.0	1.8	1.8	23.2
EZ 108	0.0	0.0	1.7	5.2	29.2
EZ 109	0.0	0.0	0.0	5.3	44.8
EZ 110	0.0	0.0	1.8	1.8	3.4
EZ 111	0.0	0.0	0.0	1.8	49.9
MEAN	**0.0**	**0.0**	**1.7**	**3.8**	**32.3**
EZ 2 Parental lines					
Female	0.0	0.0	3.4	3.4	12.1
Male	0.0	1.2	5.1	18.6	63.2

Table 4.7 – Incidence of black shank in MDDH lines of different generations of the EZ2 genotype, in five different weeks after transplant. HT Location, 2014.

Line	Weeks After Transplant				
	4th (%)	6th (%)	8th (%)	10th (%)	12th (%)
F_1 Generation DH lines					
EZ 163	0.0	0.0	1.7	13.4	35.7
EZ 164	0.0	0.0	0.0	1.8	5.3
EZ 165	0.0	0.0	0.0	40.6	70.9
EZ 166	0.0	0.0	1.6	26.3	58.3
EZ 167	0.0	0.0	5.2	58.5	87.2
EZ 168	0.0	0.0	0.0	1.8	21.0
EZ 169	0.0	0.0	0.0	0.0	3.5
EZ 170	0.0	0.0	0.0	0.0	3.8
MEAN	**0.0**	**0.0**	**1.1**	**17.8**	**35.7**
F_2 Generation DH lines					
EZ 196	0.0	0.0	0.0	0.0	0.0
EZ 197	0.0	0.0	0.0	0.0	3.5
EZ 198	0.0	0.0	0.0	1.9	9.9
EZ 199	0.0	0.0	0.0	0.0	5.0
EZ 200	0.0	0.0	0.0	0.0	1.8
EZ 201	0.0	0.0	1.7	4.2	5.9
EZ 202	0.0	0.0	0.0	0.0	3.4
MEAN	**0.0**	**0.0**	**0.2**	**0.9**	**4.2**
EZ 2 Parental lines					
Female	0.0	0.0	0.0	0.0	2.4
Male	0.0	0.0	1.8	19.0	37.6

Table 4.8 – Incidence of black shank in ADDH lines of different generations of the EZ2 genotype, in five different weeks after transplant. FC Location, 2014.

Line	Weeks After Transplant				
	4th (%)	6th (%)	8th (%)	10th (%)	12th (%)
F_1 Generation DH lines					
EZ 62	0.0	0.0	1.4	4.3	12.7
EZ 63	0.0	2.9	2.9	21.4	32.5
EZ 64	0.0	0.0	11.5	27.8	48.5
EZ 65	0.0	0.0	12.7	23.9	47.6
EZ 66	0.0	1.4	13.5	37.2	45.9
EZ 67	0.0	0.0	0.0	0.0	0.0
EZ 68	0.0	0.0	1.4	7.1	14.5
EZ 69	0.0	0.0	2.8	19.7	23.9
EZ 70	0.0	1.6	2.8	7.1	23.2
EZ 71	0.0	0.0	0.0	0.0	1.4
MEAN	**0.0**	**0.6**	**4.9**	**14.8**	**25.0**
F_2 Generation DH lines					
EZ 102	0.0	0.0	0.0	1.4	7.2
EZ 103	0.0	0.0	0.0	0.0	1.5
EZ 104	0.0	0.0	4.2	8.4	9.8
EZ 105	0.0	0.0	0.0	1.7	3.3
EZ 106	0.0	0.0	0.0	1.4	0.0
EZ 107	0.0	0.0	0.0	0.0	5.8
EZ 108	0.0	0.0	1.5	1.5	1.5
EZ 109	0.0	0.0	0.0	0.0	1.4
EZ 110	0.0	1.6	0.0	0.0	1.6
EZ 111	0.0	0.0	0.0	0.0	1.6
MEAN	**0.0**	**0.2**	**0.6**	**1.4**	**3.4**
EZ2 Parental Lines					
Female	0.0	1.5	1.5	2.9	2.9
Male	0.0	1.5	7.4	10.4	20.9

Table 4.9 – Incidence of black shank in MDDH lines of different generations of the EZ2 genotype, in five different weeks after transplant. FC Location, 2014.

Line	Weeks After Transplant				
	4th (%)	6th (%)	8th (%)	10th (%)	12th (%)
F_1 Generation DH lines					
EZ 163	0.0	0.0	4.2	8.3	29.7
EZ 164	0.0	0.0	0.0	0.0	0.0
EZ 165	0.0	1.4	10.2	34.5	47.6
EZ 166	0.0	0.0	1.4	15.7	20.7
EZ 167	0.0	1.5	8.7	32.2	38.0
EZ 168	0.0	0.0	3.0	10.4	16.0
EZ 169	0.0	0.0	0.0	0.0	0.0
EZ 170	0.0	0.0	0.0	0.0	0.0
MEAN	**0.0**	**0.4**	**3.4**	**12.6**	**19.0**
F_2 Generation DH lines					
EZ 196	0.0	0.0	0.0	0.0	0.0
EZ 197	0.0	0.0	0.0	0.0	0.0
EZ 198	0.0	0.0	0.0	0.0	0.0
EZ 199	0.0	0.0	0.0	0.0	1.4
EZ 200	0.0	0.0	0.0	1.4	1.4
EZ 201	0.0	0.0	0.0	0.0	3.0
EZ 202	0.0	0.0	0.0	0.0	2.9
MEAN	**0.0**	**0.0**	**0.0**	**0.2**	**1.2**
EZ 2 Parental Lines					
Female	0.0	1.5	1.5	2.9	2.9
Male	0.0	1.5	7.4	10.4	20.9

4.3.2 Differences between generations to induce haploids

Black Shank

Figure 4.1 displays the progression of the black shank incidence on the populations of the EZ1 genotype, averaged across locations. On average, the DH populations did not show more than 5% of plants displaying black shank symptoms at the 4th, 6th and 8th WAT, but there was a sharp rise in the disease incidence between the 8th and 12th WAT, especially for the male parent and the EZ1 – F_1 populations. In all locations (except the MDDH population at the FC location), the female parent of the EZ1 genotype was more tolerant to black shank than the mean for both the F_1 and F_2 generations at the 12th WAT. The male parent was more susceptible than both generations of DH in all four locations. This was as expected since the female line, TKF 7002H, was known to be much more tolerant to black shank than TKF 4028A12, the EZ1 male parent.

The EZ2 – F_1 DH population and the EZ2 male parent were more susceptible to race 1 black shank infection, compared to other populations of the same family (figure 4.2). Similar to the EZ1 family, the average number of plants displaying symptoms of race 1 black shank at the 8th WAT was less than 5% for each of the four populations of the EZ2 family.

Table 4.10 shows the statistics for the average incidence of black shank across the EZ1 populations for all five weeks in which disease incidence was recorded. The data displayed is the average incidence across locations of the disease shown in Tables 4.1 to 4.5. Even though the means of the EZ1 – F_1 and the EZ1 – F_2 populations at the 12th WAT were considerably different, the standard deviations were also high, which indicates that disease incidence varied within populations.

Although some of the DH lines derived from the F_1 generations were resistant to black shank, the average disease incidence of the EZ1-F_1 and the EZ2-F_1 DH lines were higher than the DH population derived from the F_2 generations (tables 4.10 and 4.11).

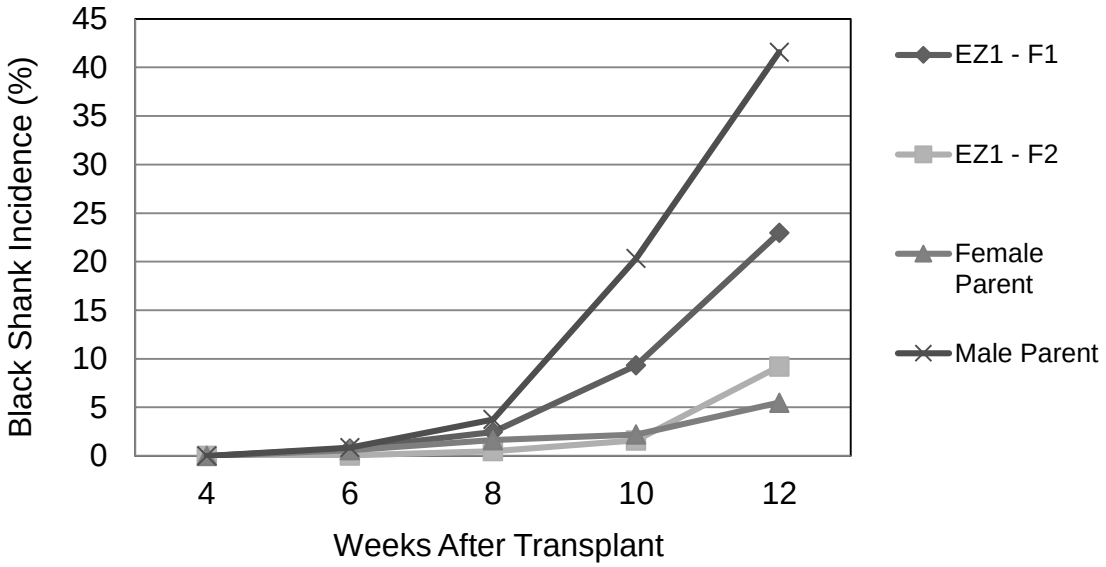

Figure 4.1 – Black shank incidence progression (%), averaged across locations, in the EZ1-F_1 and EZ1-F_2 DH populations and their parental lines. BW, HT and FC locations, 2014.

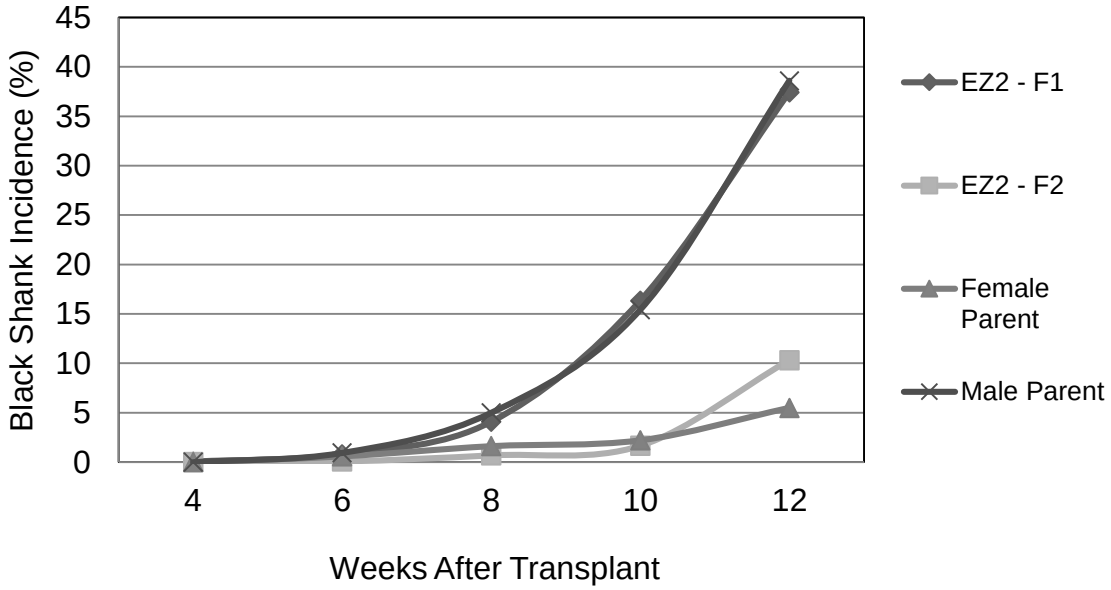

Figure 4.2 – Black shank incidence progression (%), averaged across locations, in the EZ2-F_1 and EZ2-F_2 DH populations and their parental lines. BW, HT and FC locations, 2014.

Table 4.10 – Mean, standard deviation, standard error, coefficient of variation, minimum and maximum percentage of black shank incidence in different populations of the EZ1 genotype in five different weeks after transplant. HT, FC and BW locations, 2014.

Statistics	4th WAT	6th WAT	8th WAT	10th WAT	12th WAT
EZ1 – F_1 DH population					
Mean	0	0.74	2.46	9.32	22.97
Std Dev	0	2.07	4.83	14.06	23.28
Std Error	0	0.18	0.41	1.2	1.99
CV	0	2.78	1.96	1.51	1.01
Min	0	0	0	0	0
Max	0	12.5	27.78	75	89.5
EZ1 – F_2 DH population					
Mean	0	0.05	0.48	1.6	9.21
Std Dev	0	0.49	1.71	4.35	16.9
Std Error	0	0.05	0.17	0.43	1.67
CV	0	10.15	3.57	2.73	1.84
Min	0	0	0	0	0
Max	0	5	10	30	70
TKF 7002H population (EZ1 female parent)					
Mean	0	0.59	1.62	2.21	5.48
Std Dev	0	1.86	2.61	3.98	5.53
Std Error	0	0.59	0.83	1.26	1.75
CV	0	3.16	1.62	1.81	1.01
Min	0	0	0	0	0
Max	0	5.88	5.88	11.76	15.8
TKFE 4028A12 population (EZ1 male parent)					
Mean	0	0.85	3.75	20.34	41.57
Std Dev	0	1.83	5.03	16.85	26.96
Std Error	0	0.58	1.59	5.33	8.52
CV	0	2.15	1.34	0.83	0.65
Min	0	0	0	0	0
Max	0	5	15.8	52.4	81

Table 4.11 – Mean, standard deviation, standard error, coefficient of variation, minimum and maximum percentage of black shank incidence in different populations of EZ2 genotype in five different weeks after transplant.

Statistics	4th week	6th week	8th week	10th week	12th week
----------	----------	EZ2 – F_1 DH population	----------	----------	----------
Mean	0.04	0.77	4.07	16.33	37.43
Std Dev	0.45	2.57	7	19.22	34.12
Std Error	0.04	0.23	0.62	1.71	3.04
CV	11.22	3.33	1.72	1.18	0.91
Min	0	0	0	0	0
Max	5	20	42.1	75	100
----------	----------	EZ2 – F_2 DH population	----------	----------	----------
Mean	0	0.05	0.67	1.64	10.31
Std Dev	0	0.57	2.13	4.08	17.41
Std Error	0	0.05	0.2	0.37	1.6
CV	0	10.91	3.19	2.49	1.69
Min	0	0	0	0	0
Max	0	6.25	11.11	30	73.7
----------	----------	TKF 7002H population (EZ2 female parent)	----------	----------	----------
Mean	0	0.59	1.62	2.21	5.48
Std Dev	0	1.86	2.61	3.98	5.53
Std Error	0	0.59	0.83	1.26	1.75
CV	0	3.16	1.62	1.81	1.01
Min	0	0	0	0	0
Max	0	5.88	5.88	11.76	15.8
----------	----------	TKFE 4028C5 (EZ2 male parent)	----------	----------	----------
Mean	0	0.94	5	15.42	38.6
Std Dev	0	2.06	6.29	15.25	27.66
Std Error	0	0.65	1.99	4.82	8.75
CV	0	2.19	1.26	0.99	0.72
Min	0	0	0	0	6.25
Max	0	5.88	17.65	42.1	95

The fifteen DH lines of EZ1-F_2 and the seventeen DH lines of EZ2-F_2 populations displayed similar black shank incidence, with 9.2% and 10.3%, respectively, at the 12th WAT. The F_1 generation of both genotypes displayed substantially higher levels of black shank infection, with 23% of plants affected in the twenty DH lines of the EZ1-F_1 and 37.4% for the eighteen EZ2-F_1 DH lines. As expected, the female parental line was the most resistant to black shank, followed by both pre-screened F_2 DH populations. The mean percentage of infected plants of the EZ1 - F_1 population was approximately what would be expected based on the EZ1 mid-parent (table 4.10). Conversely, the mean susceptibility to black shank of the EZ2 - F_1 population was nearly the same as observed for its male parental line (TKFE 4028C5) (table 4.11).

The analysis of variance to evaluate differences between generations within each of the genotypes revealed statistically significant differences between the F_1 and the F_2 populations for the EZ1 (table 4.12), and for the EZ2 genotypes (Table 4.13). For both genotypes, significant differences between the F_1 and the F_2 DH populations (generation) were observed for all weeks measured, except at the 4th WAT. Significant differences between black shank nurseries (locations) were observed at the 10th and at the 12th WAT for the EZ1 genotype (table 4.12), and at the 8th and at the 12th WAT for the EZ2 genotype (table 4.13).

To summarize the black shank studies, there were considerable differences in black shank susceptibility between the F_1 and F_2 derived populations. For all locations there was a clear difference in susceptibility between the F_1 and F_2-derived DH populations. Those differences were statistically significant independent of which method, ADDH or MDDH, was used to generate DH lines.

The average disease incidence across two black shank nurseries used to evaluate the F_1-derived populations of the EZ1 genotype was 28% and 22.7% for the ADDH and MDDH methods, respectively. In contrast, the disease incidences in the F_2-derived populations of the EZ1 genotype were 14% and 2.8% for the ADDH and MDDH techniques, respectively (the difference in relative survival between the two methods was due to differing

Table 4.12 – Analysis of variance of the populations of the EZ1 genotype. BW, HT and FC locations, 2014.

Period	Statistic	---------- Source of Variation ----------		
		Location	Rep(Location)	Generation
4th WAT	DF	2	7	1
	Mean Square	0	0	0
	F Value	-	-	-
6th WAT	DF	2	7	1
	Mean Square	2.8412	2.8208	31.0518
	F Value	1.12	1.11	12.23**
8th WAT	DF	2	7	1
	Mean Square	29.3032	25.5598	244.965
	F Value	2.08	1.81	17.37***
10th WAT	DF	2	7	1
	Mean Square	435.7777	91.6689	3183.568
	F Value	3.66*	0.77	26.70***
12th WAT	DF	2	7	1
	Mean Square	13536.09	73.1584	12863.36
	F Value	41.44***	0.22	39.38***

(*), (**), (***) - Differences significant at P<0.05, 0.01, 0.0001, respectively

Table 4.13 – Analysis of variance of the populations of the EZ2 genotype. BW, HT and the FC locations, 2014.

Periods	Statistics	---------- Source of Variation ----------		
		Location	Rep(Location)	Generation
4 WAT	DF	2	7	1
	Mean Square	0.1602	0.119	0.1022
	F Value	1.59	1.18	1.01
6 WAT	DF	2	7	1
	Mean Square	17.3491	1.5006	32.9848
	F Value	4.94**	0.43	9.40**
8 WAT	DF	2	7	1
	Mean Square	145.3476	4.1782	728.3755
	F Value	5.37**	0.15	26.90***
10 WAT	DF	2	7	1
	Mean Square	545.8889	204.5046	13283.68
	F Value	2.8	1.05	68.14***
12 WAT	DF	2	7	1
	Mean Square	36282.52	201.6036	46567.2
	F Value	79.11***	0.44	101.54***

(*), (**), (***) - Differences significant at P<0.05, 0.01, 0.0001, respectively

disease pressure in the nurseries used to evaluate the ADDH versus MDDH populations). The diploid female parent (TKF 7002H) had an incidence of black shank below 8% in both nurseries where the ADDH and where MDDH populations were tested. The EZ1 male parent, TKF 4028A12, displayed symptoms of black shank in more than 36% of the population across both nurseries.

A very similar trend was observed for the EZ2 genotype. Fifty percent and 27.4% of the F_1-derived DH lines were affected by black shank for the ADDH and MDDH methods, respectively. For the F_2-derived populations of ADDH and MDDH methods, 18% and 2.7% were affected, respectively. The incidence of black shank in the female parental line (TKF 7002H) was 8% and for the male parent (TKFE 4028C5), an average of 42.1% of affected plants for the ADDH nurseries and 29.3% for the lower pressure MDDH nurseries was recorded.

There was also a pronounced difference between F_1-derived versus F_2-derived lines for the percentage of plants displaying exceptionally high black shank resistance. For the F_1-derived ADDH lines from the EZ1 genotype, the incidence of black shank (averaged across three nurseries) was below 10% for only two of the ten lines. However, for the ADDH lines derived from the F_2-generation, five of the ten lines had a disease incidence below 10%. For the EZ2 genotype, only one of the 10 F_1-derived ADDH lines had an average disease incidence less than 10%, compared to two of the ten F_2-derived ADDH lines. Similar differences were observed for the MDDH derived lines. For the EZ1 genotype, only one of ten F_1-derived MDDH lines had an average disease incidence of less than 5% when averaged across a different set of three nurseries. In contrast, all five of the F_2-derived MDDH lines had a disease incidence less than 5%. For the EZ2 genotype, only 37.5% of the MDDH lines (three of eight) had less than 5% of the plants dying from black shank. In contrast, for the EZ2-F_2 derived DH population, all seven MDDH lines had 5% or lower disease incidence.

Fusarium Wilt

On average, the incidence of *Fusarium* wilt was less than 7% for all DH populations of the EZ2 and EZ3 families for any of the five weeks when data were collected. No visual symptoms were observed in the EZ2 and in the EZ3 female parent (TKF 7002H) in any of the weeks recorded. The TKFE 4028C5 and TKF 2002B6, the male parental lines of EZ2 and EZ3, respectively, displayed the highest incidence of *Fusarium* wilt, with 34.3% and 49.6% of plants infected at 12th WAT, respectively (tables 4.14 and 4.15).

Out of the ten EZ2 - F_1 DH lines tested, three did not show any symptoms of *Fusarium* wilt and only two lines had more than 10% of plants affected by the disease at the 12th WAT (table 4.14). The population of the EZ2 - F_2 generation was comprised of eight lines, among which four did not display any *Fusarium* wilt symptoms. The highest *Fusarium* wilt incidence detected was one line having 4.2% of the plants affected (table 4.14). Both F_1 and F_2 populations of the EZ3 family performed very similar to the EZ2 populations, with the exception of one EZ3-F_1 line, which had more than 25% of the plants affected by *Fusarium* wilt (table 4.15). In both EZ3 populations, 30% of the lines did not show any symptoms of the disease at the 12th WAT.

The OW nursery did not display high disease pressure, either for the selection of the haploid source plants in the 2012 growing season, or for the evaluation of the resistance of the DH lines in the season of 2014. The combination of the high resistance of the common female parent and the low disease pressure could have resulted in low incidence of *Fusarium* wilt in the DH populations. At the time plants had to be chosen to initiate the DH procedures, disease incidence was very low for both the EZ2 and the EZ3-F_1 hybrid crosses. From the 60 EZ2 – F_2 plants cultivated and used to generate DH populations in the 2012 *Fusarium* wilt nursery, only four (6.7%) displayed *Fusarium* wilt symptoms. Out of the 75 EZ3 - F_2 plants used to generate DH, only eleven (14.6%) were noticeably infected by the fungus. This level of disease incidence made it difficult to select plants with high genetic levels of *Fusarium* wilt resistance. For the EZ2-F_2 population, 93.3% of the plants displayed no *Fusarium* wilt symptoms.

Table 4.14 – Incidence of *Fusarium* Wilt in ADDH lines of different generations of the EZ2 genotype, in five different weeks after transplant. OW Location, 2014.

Line	Weeks After Transplant				
	4th (%)	6th (%)	8th (%)	10th (%)	12th (%)
F_1 Generation DH lines					
EZ 62	0.0	0.0	0.0	0.0	0.0
EZ 63	2.7	2.7	4.0	6.7	10.7
EZ 64	2.8	2.8	4.2	4.2	0.0
EZ 65	1.2	1.2	1.2	1.2	1.2
EZ 66	1.4	1.4	5.3	5.3	5.3
EZ 67	0.0	0.0	3.7	3.7	3.7
EZ 68	0.0	0.0	0.0	0.0	0.0
EZ 69	4.0	4.0	6.7	8.0	8.0
EZ 70	4.0	3.9	13.2	11.9	14.5
EZ 71	1.3	2.7	2.7	4.0	4.0
MEAN	**1.7**	**1.9**	**4.1**	**4.5**	**4.7**
F_2 Generation DH lines					
EZ 112	0.0	1.2	2.5	2.5	2.5
EZ 113	1.3	1.3	1.3	1.3	1.3
EZ 114	0.0	0.0	0.0	0.0	0.0
EZ 115	2.8	4.2	2.8	4.2	4.2
EZ 116	0.0	0.0	0.0	0.0	0.0
EZ 117	0.0	0.0	0.0	0.0	0.0
EZ 118	0.0	1.2	2.5	2.5	2.5
EZ 203	0.0	0.0	0.0	0.0	0.0
EZ 204	1.4	1.4	1.4	1.4	1.4
MEAN	**0.7**	**1.0**	**1.0**	**1.2**	**1.2**
EZ 2 Parental Lines					
Female	0.0	0.0	0.0	0.0	0.0
Male	5.0	7.5	17.8	23.8	34.3

Table 4.15 – Incidence of *Fusarium* Wilt in ADDH lines of different generations of the EZ3 genotype, in five different weeks after transplant. OW Location, 2014.

Line	Weeks After Transplant				
	4th (%)	6th (%)	8th (%)	10th (%)	12th (%)
F_1 Generation DH lines					
EZ 82	1.2	1.2	1.2	1.2	1.2
EZ 83	1.2	2.5	16.0	23.5	27.2
EZ 84	0.0	0.0	5.6	5.6	8.3
EZ 85	0.0	0.0	0.0	0.0	0.0
EZ 86	1.3	1.3	1.3	1.3	1.3
EZ 87	1.4	2.7	4.1	8.1	9.5
EZ 88	0.0	0.0	0.0	0.0	0.0
EZ 89	0.0	4.0	9.1	7.7	12.8
EZ 90	0.0	0.0	0.0	0.0	0.0
EZ 91	1.2	1.2	2.5	2.5	2.5
MEAN	**0.6**	**1.3**	**4.0**	**5.0**	**6.3**
F_2 Generation DH lines					
EZ 119	2.6	2.6	1.4	2.6	2.6
EZ 120	0.0	0.0	0.0	0.0	0.0
EZ 121	0.0	1.3	1.3	1.3	0.0
EZ 122	4.2	5.6	9.7	9.7	8.3
EZ 123	2.5	2.5	2.5	2.5	1.2
EZ 124	1.3	1.3	4.0	5.3	5.3
EZ 125	5.4	8.1	12.2	13.5	13.5
EZ 126	2.6	6.6	8.0	11.8	11.8
EZ 127	0.0	0.0	1.4	1.4	1.4
EZ 128	0.0	0.0	0.0	0.0	0.0
MEAN	**1.9**	**2.8**	**4.0**	**4.8**	**4.4**
EZ 2 Parental Lines					
Female	0.0	0.0	0.0	0.0	0.0
Male	1.2	14.3	31.1	36.4	49.6

Of these, only 10% were used to harvest anthers for induction of ADH lines, while only 16.7% of these EZ2-F_2 plants were used for production of MDH lines. Similarly, for the EZ3-F_2 population 85.4% of the plants displayed no Fusarium wilt symptoms; from these, only 8% were used to generate ADH lines, and 13.5% were used to generate MDH lines. The high resistance to *Fusarium* wilt present in the female parental line, combined with moderate pathogen pressure in the nursery during the selection in the F_2 generations (2012) and during the evaluation of the DH populations of both generations (2014) were the main factors influencing the infection and expression of disease symptoms.

Among the populations, the only substantial difference for *Fusarium* wilt incidence that was observed was for the male parental lines of both hybrid families (tables 4.14 and 4.15). The F_1 and F_2 populations of both EZ2 and EZ3 genotypes displayed high variability, as seen by the standard deviations compared to the mean infections of the populations (tables 4.16 and 4.17). Since the differences in the incidence of *Fusarium* wilt between the F_1 and the F_2 DH populations were only modest and considerable variability was observed, there were no statistically significant differences between DH generations for any of the genotypes in any of the times recorded (table 4.18).

The progression of *Fusarium* wilt in both DH populations and parental lines of the EZ2 and EZ3 genotypes over all five time intervals for which data were recorded are shown in Figures 4.3 and 4.4, respectively. The symptoms of *Fusarium* wilt for the susceptible populations started to show up more clearly in the 6^{th} WAT. The bi-weekly progression of the disease was fairly steady up to the 12^{th} WAT. This characteristic differentiates *Fusarium* wilt from black shank infection, which had a sharp rise in the infections across populations after the 8^{th} WAT, for all locations.

Table 4.16 – Mean, standard deviation, standard error, coefficient of variation, minimum and maximum percentage of *Fusarium* wilt incidence in different populations of the EZ2 genotype, measured in five different weeks after transplant at the Owensboro (OW) nursery. 2014

Statistics	4th week	6th week	8th week	10th week	12th week
----------	----------	EZ2 – F_1 DH population	----------	----------	----------
Mean	1.74	1.87	4.1	4.50	4.74
Std Dev	2.92	3.09	5.41	5.83	6.25
Std Error	0.53	0.56	0.99	1.06	1.14
CV	1.68	1.66	1.32	1.3	1.32
Min	0	0	0	0	0
Max	12	12	19.23	19.23	19.23
----------	----------	EZ 2 – F_2 DH population	----------	----------	----------
Mean	0.69	1.02	1	1.17	1.17
Std Dev	1.99	2.79	2.41	3.03	3.03
Std Error	0.41	0.57	0.49	0.62	0.62
CV	2.90	2.74	2.42	2.59	2.59
Min	0	0	0	0	0
Max	8.33	12.5	8.33	12.5	12.5
----------	----------	TKF 7002H (female parent)	----------	----------	----------
Mean	0	0	0	0	0
Std Dev	0	0	0	0	0
Std Error	0	0	0	0	0
CV	0	0	0	0	0
Min	0	0	0	0	0
Max	0	0	0	0	0
----------	----------	TKFE 4028C5 (male parent)	----------	----------	----------
Mean	5	7.52	17.76	23.8	34.26
Std Dev	1.86	3.46	12.49	14.47	10.53
Std Error	1.07	1.99	7.21	8.36	6.08
CV	0.37	0.46	0.7	0.61	0.31
Min	3.85	3.85	3.85	7.69	23.08
Max	7.14	10.71	28	35.71	44

Table 4.17 – Mean, standard deviation, standard error, coefficient of variation, minimum and maximum percentage of *Fusarium* wilt incidence in different populations of the EZ3 genotype, measured in five different weeks after transplant at the Owensboro (OW) nursery. 2014

Statistics	4th week	6th week	8th week	10th week	12th week
----------	EZ3 – F_1 DH population				----------
Mean	0.64	1.29	3.97	4.98	6.28
Std Dev	1.45	2.11	6.79	8.95	11.14
Std Error	0.26	0.39	1.24	1.63	2.03
CV	2.28	1.64	1.71	1.80	1.77
Min	0	0	0	0	0
Max	4.17	7.69	33.33	44.44	55.56
----------	EZ3 – F_2 DH population				----------
Mean	1.86	2.80	4.04	4.81	4.42
Std Dev	2.53	3.56	5.27	5.74	5.81
Std Error	0.46	0.65	0.96	1.05	1.06
CV	1.36	1.27	1.30	1.19	1.32
Min	0	0	0	0	0
Max	8.33	12.5	16.67	16.67	16.67
----------	TKF 7002H (female parent)				----------
Mean	0	0	0	0	0
Std Dev	0	0	0	0	0
Std Error	0	0	0	0	0
CV	0	0	0	0	0
Min	0	0	0	0	0
Max	0	0	0	0	0
----------	TKF 2202B6 (male parent)				----------
Mean	1.23	14.34	31.09	36.44	49.56
Std Dev	2.14	3.63	12.10	10.24	17.34
Std Error	1.23	2.09	6.99	5.91	10.01
CV	1.73	0.25	0.39	0.28	0.35
Min	0	12	20.83	29.17	32
Max	3.7	18.52	44.44	48.15	66.67

Table 4.18 - Analysis of variance of the EZ2 and EZ3 DH populations for the incidence of *Fusarium* wilt. OW, 2014.

Period recorded	Statistic	---------- Sources of variation ----------		
		Genotype	Generation	Gener(genotype)
WEEK 4	MS	0.04	0.21	36.45
	F value	0.01	0.04	6.85*
WEEK 6	MS	10.33	3.09	39.35
	F value	1.19	0.36	4.55*
WEEK 8	MS	60.31	64.88	71.04
	F value	2.12	2.29	2.50
WEEK 10	MS	119.92	86.03	70.74
	F value	2.95	2.12	1.74
WEEK 12	MS	161.55	208.33	20.69
	F value	3.00	3.87	0.38

Degrees of Freedom for all sources of variability = 1

(*), (**), (***) - Differences significant at P<0.05, 0.01, 0.0001, respectively

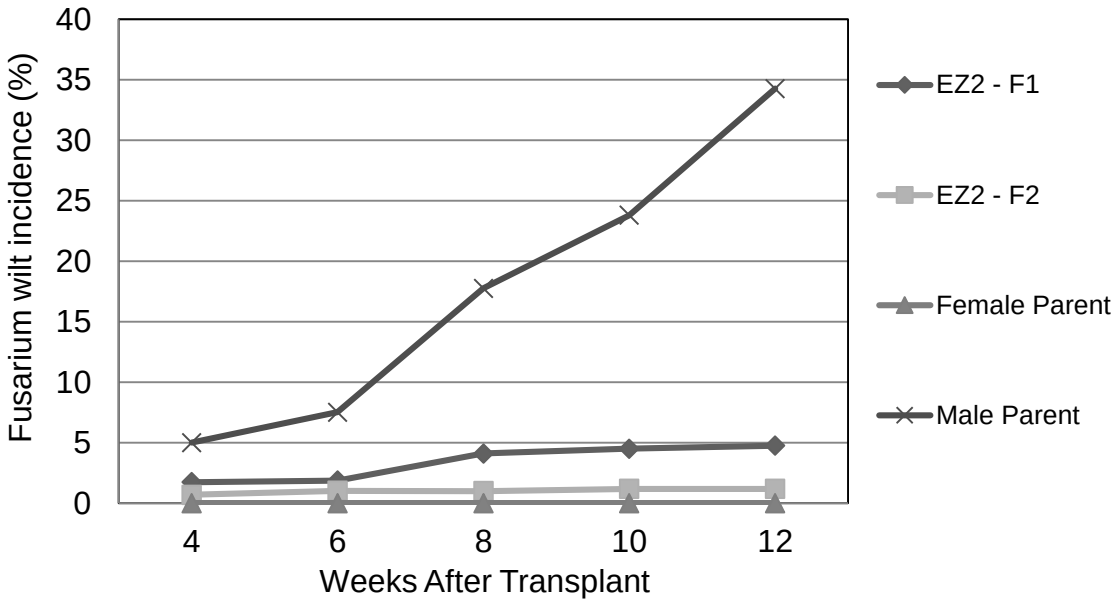

Figure 4.3 – Bi-weekly progression of the *Fusarium* wilt incidence (%) in the EZ2-F_1, EZ2-F_2 DH populations and their parental lines

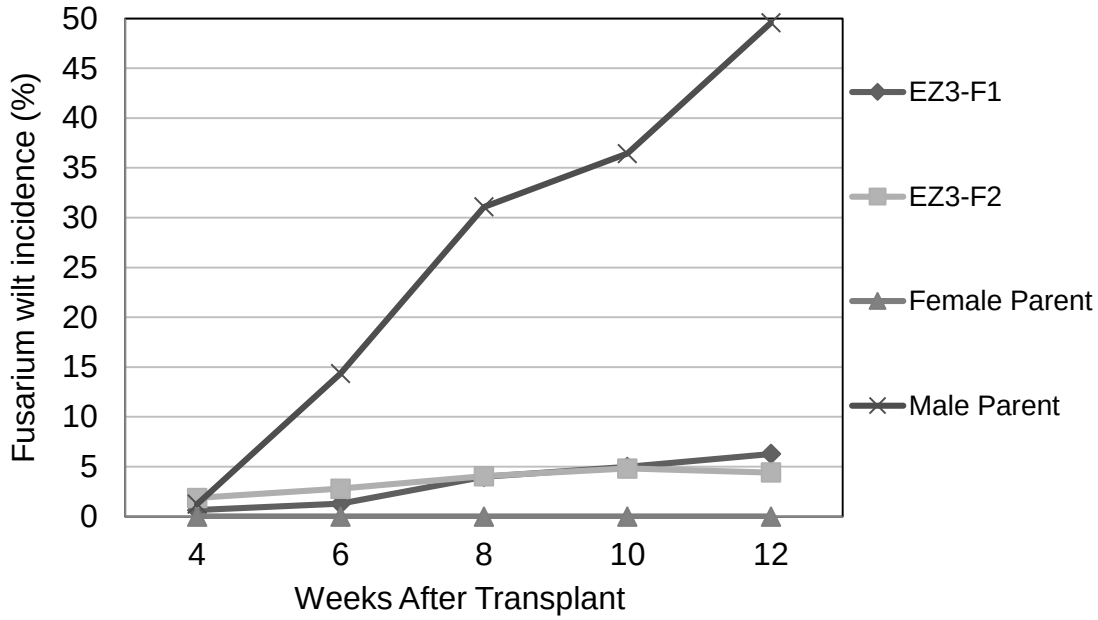

Figure 4.4 – Bi-weekly progression of the *Fusarium* wilt incidence (%) in the EZ3-F_1, EZ3-F_2 DH populations and their parental lines.

4.4 Conclusions

The nature of the pathogens, the levels of resistance inherited from the parental lines and the disease pressure in the different nurseries were important factors differentiating the response of DH populations between the black shank and *Fusarium* wilt studies. Even though the mechanism of resistance to both diseases in tobacco is controlled by several genes, the pathogens affected the plants in distinct forms. For the black shank study, the BW location displayed the highest black shank pressure and the FC location, the least. However, for all locations there was a clear difference in susceptibility between the F_1 and F_2 derived DH populations. The difference between populations followed the same trends for the *Fusarium* wilt trials, however due to the low disease pressure, the difference between generations used to derive the haploid lines was not statistically significant. One of the most prominent characteristics of both studies was the high variability within the F_1 and F_2 DH populations. There were some highly resistant lines in the F_1-derived DH populations as well as susceptible lines within the F_2-derived DH, for both black shank and the *Fusarium* wilt.

Results from the previous chapter of this dissertation showed that generating large amounts of haploids can be difficult, especially for some genotypes. By delaying the DH process until the F_2 generation, plants from a segregating population can be grown in a field nursery having high levels of disease pressure. Only plants displaying good resistance to diseases would be selected for use in the generation of haploid lines. There will be an increase in the frequency of individuals with high levels of resistance in any population, when prescreened F_2 lines are used to generate haploids instead of random F_1 lines. This is notably advantageous in situations where the generation of a large number of haploid lines is onerous and difficult. When only a limited number of haploids can be obtained, the chances of succeeding in generating useful haploid parental lines will increase as the frequency of resistant individuals in the population increases. The high frequency of individuals with high levels of resistance results in a higher number of plants available for selection for other desirable agronomic traits, therefore increasing the probability of success in the breeding process.

Delaying the DH process in tobacco from the F_1 to the F_2 generation could add up to twelve months to the development of homozygous breeding lines. However, haploids generated from F_2 pre-screened plants displayed higher levels of resistance, increasing the chance to identify superior lines having both high disease resistance and good agronomic characteristics. The delay in the process may be offset by having to screen fewer finished DH lines to identify superior lines having both high disease resistance and good agronomic characteristics. This is particularly true in cases where the overall objective is to combine high quantitatively inherited resistance to soil-borne disease with qualitatively inherited resistance to multiple other diseases.

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